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Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease (MPD) characterized by the expression of the *p210-BCR/ABL* fusion gene [1]. This gene is produced by the reciprocal translocation (9; 22) (q34; q11) that juxtaposes the 3'end of Abelson leukemia virus (ABL) gene with the 5' end of the breakpoint cluster region (Bcr) gene on chromosome 22. The transcript formed as a result encodes for the BCR/ABL fusion protein with constitutively active tyrosine kinase activity [2-6]. Recent studies with inducible BCR/ABL transgenic mice showed that expression of BCR/ABL in hematopoietic stem cells and progenitors (HSC/P) is required and is sufficient to induce MPD [7]. If untreated, chronic phase (CP) CML patients progress to a poor-prognosis myeloid or lymphoid blastic phase (BP). The only curative treatment for CML is allogeneic HSC transplantation. The long-term survival rate for this procedure is approximately 65%, however, the procedure is only available to a minority of CML patients due to a lack of compatible donors and age [8-10]. Imatinib is an ABL kinase inhibitor that shows significant activity in CP CML and Ph-positive acute leukemias [11]. By selective induction of apoptosis of BCR/ABL-positive cells [12-14], it provides an effective treatment in CML and has rejuvenated the field of rationalized drug design. The selective inhibitory activity of imatinib toward BCR/ABL has been associated with three problems: the emergence of BCR/ABL mutants in the kinase domain that confer resistance to imatinib; the evidence that CML stem cells are the least vulnerable to ABL-targeted therapy and may serve as reservoirs for occult CML progression; and the relatively low impact of imatinib therapy on the outcome of BP CML patients [15-18]. Resistance to imatinib has an incidence of 4% annually [19]. Different mutated residues have been reported [20], with mutations of Y253, E255, T315, and M351 giving rise to approximately 60% of resistance-conferring mutations in patients at the time of relapse. Efforts to overcome mutant-derived imatinib resistance have led to the development of newer generations of ABL-kinase inhibitors (dasatinib, nilotinib, PD166326, etc.) that are ATP-binding independent. The utility of ABL kinase based rationalized drug design has been demonstrated in recent studies [21-23] that showed that dasatinib and nilotinib are effective against imatinib-resistant BCR/ABL mutants. However, the relatively frequent mutation T315I is insensitive to all the tested tyrosine kinase inhibitors and require alternative therapeutic strategies [24-26]. In addition, many patients present with persistent, BCR/ABL expression in HSC/P [27], also called residual disease and once patients fall into BP CML, their clinical

outcome practically remains unchanged compared with the pre-imatinib era [17]. An alternative to bypass the imatinib-resistance is to target other signaling components downstream of BCR/ABL. During the first year of this award, we have moved forward in the analysis of the role of Rac GTPases in chronic-phase and blastic-phase CML. As a result of this work, one original manuscript in the journal Cancer Cell and two reviews on the topic in the journals Leukemia and Methods in Enzymology have been published.

Body

Rac is hyperactivated in chronic-phase CML HSC/P

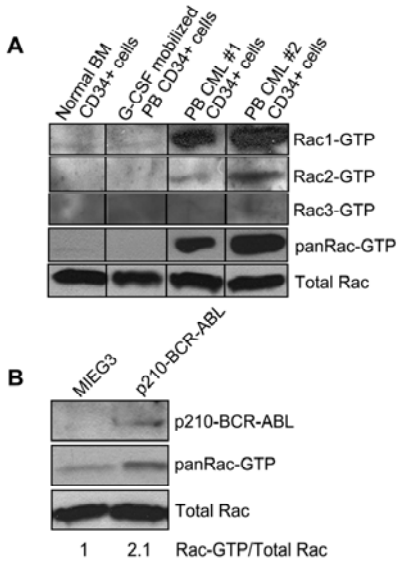

Figure 1. Rac GTPases are hyperactivated in chronic-phase human CML HSC/P and murine HSC/P expressing p210-BCR-ABL. A. Primary human CD34+ BM or G-CSF-mobilized peripheral blood cells and CD34+ chronic-phase CML peripheral blood cells (from two different CML patients) were starved for 1 hour and analyzed for Rac activation in a PAK binding domain (PBD) pull-down assay. Samples were blotted and analyzed for Rac1-GTP, *Rac2*-GTP, Rac3-GTP, total Rac-GTP and total Rac protein content. B. 5-FU-treated murine LDBM cells were transduced with either MIEG3 or MSCV-p210-BCR-ABL, bicistronic vectors expressing EGFP. The EGFP+ cells were sorted, serum-starved for six hours, stimulated with 100 ng/ml SDF-1a for five minutes, lysed, and used in a PAK binding domain (PBD) pull-down assay. The ratio of GTP-bound Rac to total Rac was determined by densitometry. The data represent one of three experiments with similar results.

Recent studies in cell lines have suggested that Rho GTPases can be activated by p210-BCR-ABL *in vitro* and *in vivo* [28, 29]. Since the expression of p210-BCR-ABL in HSC appears to be sufficient to induce a transformation phenotype, we first analyzed whether Rac isoforms were hyperactivated in human chronic phase CML HSC/P. Activation of Rac was determined by p21-activated kinase (PAK) binding domain (PBD) pull-down assays in isolated CD34+ cells from CML patients. We observed that Rac1, Rac2, and, to a lesser degree, Rac3 were hyperactivated in $CD34^+$ cells purified from peripheral blood of two CML patients at diagnosis (**Figure 1A**). To determine the effect of p210-BCR-ABL expression on activation of the Rac subfamily of Rho GTPases in a murine model of p210-BCR-ABL disease, we exogenously expressed p210-BCR-ABL in primary murine HSC/P. 5-fluorouracil (5-FU)-treated low density BM (LDBM) cells were transduced with bicistronic vectors expressing enhanced green fluorescent protein (EGFP) either alone (empty vector, MIEG3) or with p210-BCR-ABL (MSCV-p210-BCR-ABL)[30]. Sorted, $EGFP^+$ cells were starved and then stimulated with stromal-derived factor-1α (SDF-1α), a chemokine known to induce migration of p210-BCR-ABL-expressing HSC/P [31]. Expression of p210-BCR-ABL in LDBM cells, confirmed by immunoblot, led to a >2-fold increase in GTP-bound Rac (**Figure 1B**) compared to MIEG3 transduced cells.

Rac1 and Rac2 deficiency significantly attenuates p210-BCR-ABL-mediated MPD *in vivo*

To determine whether and which Rac GTPases are required for the development of p210-BCR-ABL-induced leukemogenesis *in vivo*, we utilized gene-targeted mice deficient in *Rac2* and with conditional (floxed) alleles of *Rac1* in a retroviral murine model of CML. 5-FU-treated Cre^{Tg+};WT, Cre^{Tg+};$Rac1^{flox/flox}$, Cre^{Tg+};$Rac2^{-/-}$, and Cre^{Tg+};$Rac1^{flox/flox}$;$Rac2^{-/-}$ LDBM cells were transduced with MIEG3 (control, empty vector) or MSCV-p210-BCR-ABL and sorted for $EGFP^+$ expression. Irradiated C57Bl/6 mice were transplanted with 50,000-75,000 of the $EGFP^+$ transduced cells together with 500,000 unmanipulated BM cells to assure the rescue of normal hematopoiesis in the post-irradiation period. Ten days post-transplant, recipient mice were treated with polyI:C as previously described [32, 33] to delete floxed *Rac1* genomic sequences (hereafter designated $Rac1^{\Delta/\Delta}$ mice). Recipient mice transplanted with MSCV-p210-BCR-ABL-transduced Cre^{Tg+};WT or Cre^{Tg+};$Rac1^{flox/flox}$ LDBM cells that were treated with PolyI:C

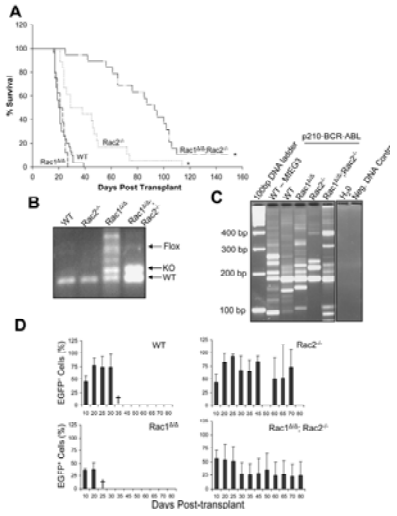

Figure 2. Rac GTPases are critical regulators of p210-BCR-ABL-mediated leukemogenesis. A. Kaplan-Meier survival curve of mice that were transplanted with MSCV-p210-BCR-ABL-transduced wild type and Rac-deficient cells. Mice demonstrating engraftment (monitored by the percentage of EGFP+ cells in the peripheral blood) of less than 1% at two consecutive time points were censored from the study. Genotypes are abbreviated in all figures as: WT: wild-type or flox allele at *Rac1* and *Rac2* loci, n=30; *Rac2-/-*: wild-type or flox allele at *Rac1* and null allele at *Rac2* locus, n=18; *Rac1*Δ/Δ: Cre-mediated null allele at *Rac1* locus and wild-type allele at *Rac2* locus, n=8; *Rac1*Δ/Δ;*Rac2-/-*: Cre-mediated null allele at *Rac1* locus and null allele at *Rac2* locus, n=19. * $p < 0.001$ (log P rank test) between MSCV-p210-BCR-ABL-expressing WT and *Rac1*Δ/Δ recipient mice and either BCR-ABL-transduced *Rac2-/-* or *Rac1*Δ/Δ;*Rac2-/-* groups. B. Representative PCR showing deletion of the *Rac1* floxed gene in the peripheral blood of *Rac1*Δ/Δ and *Rac1*Δ/Δ;*Rac2-/-* recipient mice seventy days post-transplant, as visualized by the presence of the knock-out (KO) band in the representative *Rac1*Δ/Δ and *Rac1*Δ/Δ;*Rac2-/-* animals. The presence of the wild type (WT) allele in these mice does not signify expression of the *Rac1*flox/flox allele but rather represents contribution from unmanipulated BM cells co-injected with BCR-ABL-transduced cells. Thus, the efficiency of loss of exon 1 of *Rac1* can be determined by comparing the Flox band and the KO band in these samples. C. LAM-PCR amplifying retroviral vector insertion sites in p210-BCR-ABL-expressing BM cells from mice reconstituted with wild-type, *Rac1*Δ/Δ, *Rac2-/-* and *Rac1*Δ/Δ;*Rac2-/-* cells. 3/3 leukemic *Rac1*Δ/Δ;*Rac2-/-* animals tested demonstrated oligoclonal integration patterns. D. Percentage of EGFP+ cells in the peripheral blood of all surviving mice monitored over the course of the transplant. Data represent mean ± SD of all the mice included in Fig. 2A.

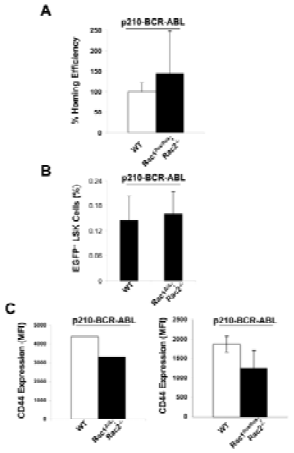

Figure 3. Homing, engraftment and CD44 expression appear not to be impaired by Rac deficiency in p210-BCR-ABL-expressing HSC/P. **A.** Expression of the *Rac1*flox alleles and deletion of *Rac2* do not appear to inhibit homing and engraftment of BCR-ABL-expressing cells. p210-BCR-ABL-expressing (EGFP+) WT and *Rac1*flox/flox;*Rac2-/-* cells were labeled with PKH26. Sorted PKH26+/EGFP+ cells were transplanted into lethally irradiated C57Bl/6 mice. Sixteen hours later, mice were sacrificed and the number of PKH26-labeled cells in the bone marrow was determined. Data represent mean ± SD, n = 3-7 mice per group, p=N.S. **B.** Short-term engraftment of EGFP+Lin-c-Kit+Sca-1+ bone marrow HSC/P harvested from mice transplanted with p210-BCR-ABL-expressing WT or *Rac1*D/D;*Rac2-/-* BM cells 18 days post-transplant. Data represent mean ± SD, n = 4 mice per group. **C & D.** CD44 expression was analyzed in p210-BCR-ABL-expressing cells *in vitro* and *in vivo*. **C.** Transduced and sorted p210-BCR-ABL (p210-BCR-ABL-EGFP) and (Cre-YFP) WT and *Rac1*Δ/Δ;*Rac2–/–* BM cells were analyzed for CD44 expression. **D.** p210-BCR-ABL-transduced WT and *Rac1*flox/flox, *Rac2–/–* BM cells were labeled with PKH26. PKH26+/EGFP+ BM cells were transplanted into lethally irradiated recipients and retrieved from femorae and tibiae after 16 hours post-transplantation. CD44 expression was analyzed on PKH26+/EGFP+ homed BM cells (p=N.S.). Data represent mean ± SD, n=3 mice per group.

uniformly developed CML-like MPD (leukocytosis, splenomegaly, pulmonary hemorrhage, and extensive liver infiltration with hematopoietic cells at necropsy) and died within forty days post-transplant (**Figure 2A**), consistent with the MPD phenotype, while all of the mice transplanted with MIEG3-transduced WT or $Rac1^{\Delta/\Delta}$ cells were still alive at day 100 post-transplant (**Table 1**). Strikingly, mice transplanted with p210-BCR-ABL-expressing Cre^{Tg+};$Rac1^{flox/flox}$;$Rac2^{-/-}$ cells that were treated with polyI:C to delete *Rac1* in the *Rac2*-null background (designated $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice) showed significantly prolonged survival (**Figure 2A**, $p < 0.001$). Nearly 50% of these mice were still alive 100 days post-transplant. PCR analysis confirmed loss of exon 1 of *Rac1* in $Rac1^{\Delta/\Delta}$ and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice treated with polyI:C (**Figure 2B**). Clonal analysis by linear amplification-mediated polymerase chain reaction (LAM-PCR) of BM cells from leukemic mice showed similar and oligoclonal reconstitution of p210-BCR-ABL-expressing wild-type, $Rac1^{\Delta/\Delta}$, $Rac2^{-/-}$ and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ cells (**Figure 2C**), suggesting that the delay in disease progression in these animals was not due to loss of p210-BCR-ABL vector integration and expression. Southern blot analysis confirmed the LAM-PCR study, showing 1-3 major clones/leukemia and no differences in the number of clones between genotypes (data not shown). All recipient mice maintained peripheral $EGFP^+$ cells throughout the study, confirming sustained engraftment of p210-BCR-ABL-expressing cells even in the absence of Rac1 and Rac2 (**Figure 2D**), a finding that is noteworthy due to our previous observations that hematopoietic engraftment of $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ HSC/Ps is rapidly lost in the

Table 1:

Recipient Mice	Survival at Day 100	Spleen weight	Pulmonary Hemorrhage
WT MIEG3	24/24	0.089 ± 0.03	0/24
Rac1$^{\Delta/\Delta}$ MIEG3	8/8	0.176 ± 0.17	0/8
Rac2$^{-/-}$ MIEG3	20/21	0.102 ± 0.04	0/21
Rac1$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ MIEG3	13/14	0.131 ± 0.08	0/14
WT BCR-ABL	0/30	0.410 ± 0.12	17/18
Rac1$^{\Delta/\Delta}$ BCR-ABL	0/8	0.333 ± 0.06	8/8
Rac2$^{-/-}$ BCR-ABL	1/18	0.553 ± 0.19	12/13
Rac1$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BCR-ABL	8/19	0.312 ± 0.17	4/16

absence of p210-BCR-ABL [33]. Survival of mice transplanted with p210-BCR-ABL-transduced *Rac2*$^{-/-}$ cells was intermediate to *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice and significantly longer ($p < 0.001$) than the WT and *Rac1*$^{\Delta/\Delta}$ mice (**Figure 2A**).

We next confirmed that the increased survival seen in Rac-deficient, p210-BCR-ABL-expressing mice was not related to defective engraftment of Rac-deficient HSC. To analyze the homing and engraftment of p210-BCR-ABL-expressing cells in the presence or absence of the Rac GTPases, recipient mice were transplanted with PKH26-labeled WT and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ donor BM cells transduced with p210-BCR-ABL. The percentages of PKH26/EGFP-expressing cells in BM at 16 hours post-transplantation were similar between the WT and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ animals, suggesting unimpaired homing despite lack of Rac2 expression (**Figure 3A**), consistent with our previous findings in normal hematopoietic cells [33]. In addition, there was an equivalent frequency of EGFP^{+}Lin^{-}Sca1^{+}c-Kit^{+} cells observed in the BM of recipient mice transplanted with either WT or *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ p210-BCR-ABL-expressing cells 18 days post-transplant after deletion of *Rac1* sequences, suggesting that Rac-deficient HSC/P maintain an early graft as efficiently as wild type cells in the presence of p210-BCR-ABL (**Figure 3B**). In agreement with these data, there was no significant difference in expression of the hyaluronan receptor, CD44, which has recently been shown to play a specific and essential role in the homing and engraftment of p210-BCR-ABL-expressing leukemia-initiating cells [34] either *in vitro* (**Figure 3C)**, using co-transduced (Cre-YFP and p210-BCR-ABL-EGFP) and sorted *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ LDBM cells, or *in vivo* (**Figure 3D**), from animals injected with p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ cells, compared to p210-BCR-ABL-expressing wild type cells. Thus, these data suggest that prolonged survival of mice transplanted with p210-BCR-ABL-transduced Rac-deficient cells is not explained by reduced engraftment of leukemia-initiating stem cells.

Phenotype of MPD in the Rac-deficient mice

p210-BCR-ABL-expressing WT mice rapidly developed significant EGFP+ leukocytosis that persisted until death (**Figures 2D and 4A**). p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$ mice succumbed to splenomegaly and pulmonary hemorrhage (**Table 1**), consistent with the MPD phenotype. The majority of p210-BCR-ABL *Rac2*$^{-/-}$ mice showed gradual progression of leukocytosis and eventually died of MPD (**Figure 4A and Table 2**). Differential counts of the peripheral blood from p210-BCR-ABL-expressing WT and *Rac2*$^{-/-}$ mice ~30 days post-transplant demonstrated neutrophilia and the presence of immature granulocyte precursors and blasts in the peripheral blood (**Figures 4B and 4C**), consistent with the MPD previously described in this model [5, 35]. As expected, development of leukemia in these mice was accompanied by a predominance of EGFP^{+} cells in the blood (**Figure 2D**), BM and spleen (data not shown). EGFP^{+} cells in the spleen and BM of all p210-BCR-ABL-transplanted WT, *Rac1*$^{\Delta/\Delta}$, and *Rac2*$^{-/-}$ recipient mice were uniformly Gr-1^{+}/Mac-1^{+} (**Table 2**). In contrast to these mice, p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ recipient mice showed normal peripheral blood morphology at ~30 days post-transplant, in spite of significant chimerism with EGFP^{+} p210-BCR-ABL-expressing cells (**Figures 2D, 4B, and 4C**). The few p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice that developed early disease (arbitrarily defined as ≤69 days; 4/19 *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice) had either a myeloid (Gr-1^{+}/Mac-1^{+}, 67% of three animals tested) or lymphoid (B220^{+}, 33% of three animals tested) phenotype. One of the mice with a myeloid phenotype developed a solid tumor in the spine that showed high (86%) EGFP expression. The mice that

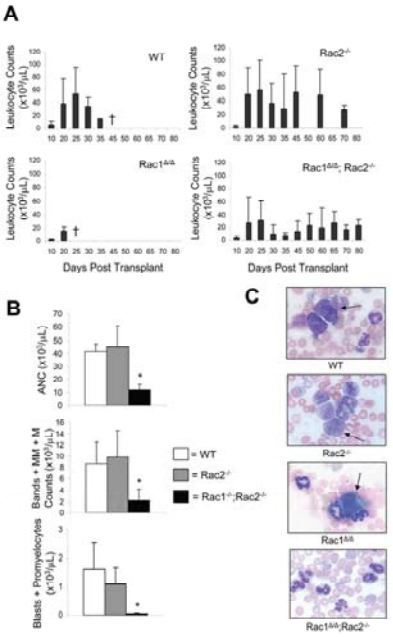

Figure 4. Deficiency of Rac1 and Rac2 expression significantly delays or appreciably inhibits the development of leukocytosis in BCR-ABL-expressing recipient mice. **A.** Time course showing average leukocyte counts in the peripheral blood of recipient mice that were injected with either WT or Rac-deficient LDBM cells transduced with MSCV-p210-BCR-ABL. WT n=30; *Rac2-/-* n=18; *Rac1*Δ/Δ n=8; *Rac1*Δ/Δ;*Rac2-/-* n=19. Data represent mean ± SD. Crosses represent time points at which no animals were surviving for analysis. **B.** Differential counts show decreased frequency of blasts and other immature myeloid progenitors in the peripheral blood of *Rac1*Δ/Δ;*Rac2-/-* BCR-ABL-transduced recipient mice approximately 30 days post-transplant, compared to the WT and *Rac2-/-* mice. Data represent mean ± SD. ANC: average neutrophil counts; MM: metamyelocytes; M: myelocytes. WT n=7; *Rac2-/-* n=7; *Rac1*Δ/Δ;*Rac2-/-* n=8. *p < 0.05 between BCR-ABL-expressing WT and *Rac2-/-* mice and the BCR-ABL-transduced *Rac1*Δ/Δ;*Rac2-/-* group. **C.** Morphology of cells present in peripheral blood smears from representative BCR-ABL-transduced WT and Rac-deficient recipient mice approximately 30 days post-transplant. Myeloblasts (arrows) were apparent in all mice except for BCR-ABL-recipient *Rac1*Δ/Δ;*Rac2-/-* mice. Bars = 10 mm.

succumbed to late disease (≥ 70 days; 15/19 $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice) had either a myeloid (Gr-1$^+$/Mac-1$^+$, 62% of 13 animals tested), lymphoid (B220$^+$, 23% of 13 animals tested), or bi-lineage (myeloid and lymphoid; 15% of 13 animals tested) phenotype. Additionally, two of the p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice with late onset disease developed solid tumors in the skull and brain, with histochemical and histological features consistent with CD68$^+$ histiocytic sarcoma. Two of the p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice developed solid tumors in the spine with high (> 70%) EGFP expression. One of the animals with a high percentage (40%) of CD3$^+$ cells also presented with a tumor on the left kidney.

Activation of signaling cascades in transformed cells of p210-BCR-ABL-expressing mice

These data show that loss of Rac1 and Rac2 expression plays a key role in attenuation of the MPD phenotype, but suggest that late molecular events may overcome the loss of Rac1 and Rac2 function. To assess the status of Rac activation in p210-BCR-ABL-expressing WT, $Rac1^{\Delta/\Delta}$, $Rac2^{-/-}$, and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ mice developing MPD, we performed PBD pull-down assays on splenocytes of diseased animals. Although *Rac1* and *Rac2* gene deletion was confirmed by PCR analysis (data not shown), active GTP-bound Rac as detected by a pan-Rac antibody was elevated in all p210-BCR-ABL-expressing leukemic mice tested (**Figure 5A**), suggesting that the third member of the Rac subfamily, Rac3, may be activated in p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ leukemic mice. Rac3 expression was confirmed in all leukemic animals tested by immunoblot (**Figure 5B**, lower panel) and PBD pull-down assays of splenocytes harvested from additional diseased animals showed enhanced GTP-bound Rac3 in p210-BCR-ABL-expressing leukemic mice, most clearly in the $Rac2^{-/-}$ and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ samples (**Figure 5B**, upper panel), suggesting that Rac3 likely plays a key role in the eventual development of CML-like MPD.

Activation of Rac by p210-BCR-ABL in the WT mice was associated with increased baseline ERK, JNK, p38, Akt, STAT5, and CrkL phosphorylation (**Figure 5C**). These results are consistent with Rac signaling pathways previously implicated by us and others [32, 36]. Activation of ERK, JNK, p38, and CrkL was similar to WT mice in $Rac1^{\Delta/\Delta}$ mice, but was reduced in $Rac2^{-/-}$ and nearly completely abrogated in $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ splenocytes harvested from mice that developed MPD, despite continued activation of BCR-ABL in p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ splenocytes as determined by phospho-tyrosine immunoblots and increased activation of Rac3 (**Figures 5B and 5D**). These biochemical findings are strikingly in parallel with the survival curves of p210-BCR-ABL-expressing mice. Phosphorylation of Akt was reduced in all of the Rac-deficient mice compared with WT mice. These data strongly suggest that activation of multiple signaling pathways by p210-BCR-ABL is dependent on Rac1 and Rac2. Interestingly, STAT5 phosphorylation was

Table 2:

Mouse genotype	Days Post-transplant Leukemia First Observed	Phenotype/ % Observed
WT BCR-ABL	16	Myeloid (Gr-1/Mac-1) 100%
Rac1$^{\Delta/\Delta}$ BCR-ABL	17	Myeloid (Gr-1/Mac-1) 100%
Rac2$^{-/-}$ BCR-ABL	21	Myeloid (Gr-1/Mac-1) 100%
Rac1$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BCR-ABL—early disease	25	Myeloid (Gr-1/Mac-1) 67% Lymphoid (B220) 33%
Rac1$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BCR-ABL—late disease	70	Myeloid (Gr-1/Mac1) 62% Lymphoid (B220) 23% Myeloid/Lymphoid 15%

variably diminished in leukemic splenocytes even in the absence of Rac2 and more severely reduced but still detectable in the absence of both Rac1 and Rac2. p210-BCR-ABL thus may mediate activation of this pathway via induced Rac3 and/or STAT5 may be activated independently of Rac GTPases.

Rac is a molecular target in p210-BCR-ABL-expressing cells

These results strongly support the hypothesis that p210-BCR-ABL signaling is dependent on Rac activation, suggesting that Rac GTPases may be unique molecular targets for CML therapy. We next used a genetic approach to determine whether deficiency of Rac1 and Rac2 influences p210-BCR-ABL-induced hyperproliferation of hematopoietic cells *in vitro*, a characteristic of this retroviral model of p210-BCR-ABL expression. LDBM cells were harvested from *Rac1*$^{flox/flox}$ and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ mice and co-transduced with MSCV-Cre-YFP and either MIEG3 or MSCV- p210-BCR-ABL. Deletion of *Rac1* in the Cre-YFP-expressing cells was confirmed by PCR analysis (data not shown). Proliferation of sorted EGFP^{+} (*Rac1*$^{flox/flox}$) and EGFP^{+}/YFP^{+} (*Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$) cells was determined by thymidine incorporation and cell counts. As shown in **Figure 6A**, p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$ primary hematopoietic cells displayed significantly increased proliferation, compared to MIEG3-transduced *Rac1*$^{flox/flox}$ cells. *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ cells exhibited significantly reduced p210-BCR-ABL-mediated hematopoietic cell proliferation, compared to p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$ cells, suggesting that p210-BCR-ABL-mediated hyperproliferation of hematopoietic cells *in vitro* is dependent on activation of Rac1 and Rac2 and further validating Rac GTPases as key regulators of p210-BCR-ABL-mediated MPD.

To examine if pharmacologic inhibition of Rac in the presence of p210-BCR-ABL leads to attenuated cell proliferation, WT LDBM cells transduced with either MSCV-p210-BCR-ABL or MIEG3 and sorted for EGFP^{+} expression were serum starved and incubated for 48 hours in the presence of increasing concentrations of NSC23766, a Rac-specific small molecule inhibitor that has been shown to inhibit Rac1 and Rac2 [32, 33], and we have found to inhibit Rac1, Rac2, and Rac3 in BCR-ABL-transduced LDBM cells (see **Figure 7)**. NSC23766 does not inhibit RhoA or Cdc42 [37]. Although inhibition of Rac activation with NSC23766 is reversible and less complete than genetic deletion, p210-BCR-ABL-induced proliferation was inhibited in a dose-dependent manner (**Figure 6B, solid bars**). No inhibition of MIEG3-transduced cells was detected at the same concentrations of inhibitor (**Figure 6B, empty bars**). Since we hypothesized that Rac3 was abnormally activated in splenocytes harvested from p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice developing late MPD, the effect of the Rac-specific inhibitor NSC23766 was tested *in vitro* on BM cells derived from these mice. The proliferation of EGFP^{+} *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BM cells harvested from *Rac1*;*Rac2*-deficient mice with MPD was inhibited in a dose-dependent manner by the Rac inhibitor (**Figure 6C**), and immunoblotting confirmed inhibition of Rac3 activation in leukemic samples harvested from *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ animals (data not shown). These results further implicate Rac3 in the late development of MPD in the *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice.

To determine the effect of Rac inhibition on proliferation of an Imatinib-resistant p210-BCR-ABL mutant [38, 39], Ba/F3 cells expressing the highly resistant T315I-p210-BCR-ABL mutant were incubated with Imatinib and/or NSC23766. As reported previously [26], addition of up to 3 μM Imatinib alone had little effect on proliferation of these cells (less than 10% inhibition,

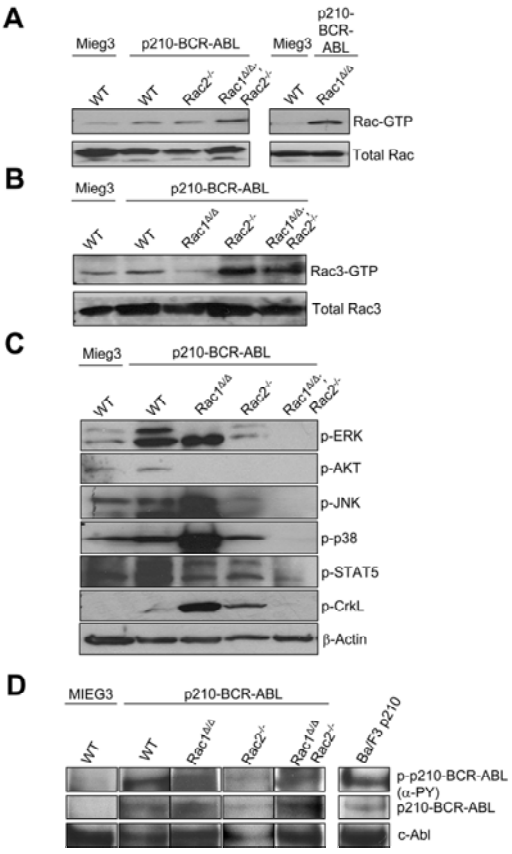

Figure 5. Rac GTPases are implicated in BCR-ABL mediated activation of multiple signaling cascades. **A.** Representative example of Rac activation (PBD) pull-down assays performed on splenocytes harvested from MIEG3 and MSCV-p210-BCR-ABL-transduced WT and Rac-deficient mice developing MPD. Top panel (Rac-GTP) represents activated total Rac, lower panel represents total Rac protein expressed by immunoblot. **B.** Representative example of PBD pull-down assays of splenocytes harvested from leukemic animals to monitor Rac3 activation, using a Rac3-specific antibody. **C.** Representative examples of immunoblot analyses of splenocytes from BCR-ABL-expressing WT and Rac-deficient recipient mice developing MPD using phospho-antibodies specific to ERK, JNK, p38, Akt, CrkL, and STAT5. b-actin was used as a loading control..For each analysis, a minimum of three specimens from different mice were analyzed with similar results. **D**. Activation of p210-BCR-ABL in BM cells harvested from deceased or sacrificed leukemic mice. Phospho-p210-BCR-ABL expression is demonstrated in leukemic WT, *Rac1*Δ/Δ, *Rac2*-/-, and *Rac1*Δ/Δ;*Rac2*-/- BM cells with a phospho-tyrosine antibody. A minimum of 3 samples in each genotype confirmed phosphorylation of p210-BCR-ABL in these samples. Expression of total p210-BCR-ABL and c-Abl was visualized using a c-Abl antibody. As a positive control, lysates from Ba/F3 cells stably expressing p210-BCR-ABL were analyzed.

Figure 6D).

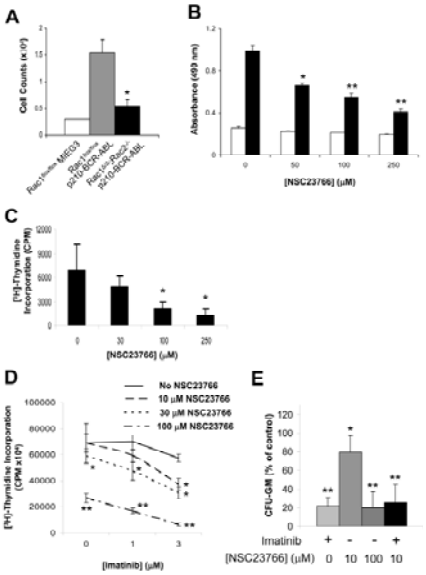

Figure 6. Loss of Rac activation via gene deletion or by treatment with NSC23766, a Rac-specific inhibitor, diminishes the proliferation of BCR-ABL-expressing murine and human cells. **A.** 5-FU-treated *Rac1*flox/flox and *Rac1*flox/flox;*Rac2*-/- HSC were transduced with MSCV-Cre-YFP to delete the *Rac1* floxed genomic sequence, together with MIEG3 or MSCV-p210-BCR-ABL. The EGFP+ and EGFP+/YFP+ cells were sorted and plated. Cell proliferation was determined by cell counts 48 hours after the cells were plated. Data represent mean ± SD, n=3 in each of two independent experiments. * $p < 0.05$ between the BCR-ABL-transduced *Rac1*flox/flox cells and the MIEG3-expressing *Rac1*flox/flox and BCR-ABL-expressing *Rac1*Δ/Δ;*Rac2*-/- cells. **B.** 5-FU treated WT murine LDBM cells were transduced with either MIEG3 (white bars) or MSCV-p210-BCR-ABL (black bars), and the EGFP+ cells were sorted. Cells were then plated in the presence of increasing concentrations of NSC23766, and proliferation was determined 48 hours later by MTS assay. Data represent mean ± SD, n=3 for three independent experiments. * $p < 0.01$, ** $p < 0.001$ vs no drug. **C.** Effect of NSC23766 on BM cells harvested from BCR-ABL-expressing *Rac1*Δ/Δ;*Rac2*-/- mice that developed MPD. Results are representative of mean ± SD from proliferation assays performed on three different *Rac1*Δ/Δ;*Rac2*-/- mice in triplicate. *$p < 0.05$ vs no drug. **D.** Effect of increasing concentrations of NSC23766 alone and in combination with Imatinib on the proliferation of Imatinib-resistant Ba/F3 p210-BCR-ABL-T315I cells. Data represent mean ± SD of four independent assays per sample done in duplicate. * $p < 0.01$; ** $p < 0.001$. E. Analysis of CFU-GM formation from CML patients that developed myeloid blast crises in the presence of 1 mM Imatinib (n=10 patients), NSC23766 (n=10 patients), or a combination of Imatinib and NSC23766 (n=5 patients). Data represent mean ± SD of the level of inhibition reached for each specific specimen. * $p < 0.01$; ** $p < 0.001$.

However, T315I-expressing cells treated with NSC23766 alone or in combination with Imatinib showed >90% inhibition of proliferation, similar to Ba/F3 cells expressing non-mutated p210-BCR-ABL (**Figure 6D**). Finally, the effect of NSC23766 on the growth of primary BM cells from CML patients in blast crisis was evaluated. Addition of 10-100 μM NSC23766 inhibited 20-78% of blastic phase CML BM colony forming unit-granulocyte/macrophage (CFU-GM) colonies, a response similar to 1 μM Imatinib (**Figure 6E**). Similar inhibition was observed for CML blast phase erythroid progenitor cells (data not shown), while 100 μM NSC23766 but not lower doses inhibited normal human progenitor BM cells in a fashion similar to 1 μM Imatinib (**Figures 8A and 8B**). These data demonstrate that Rac is essential for p210-BCR-ABL-induced proliferation of primary cells and pharmacologic inhibition of Rac significantly reduces p210-BCR-ABL-mediated proliferation even in the presence of highly resistant kinase mutants.

To determine the effect of NSC23766 *in vivo*, mice were transplanted with p210-BCR-ABL-expressing murine HSC/P as described above and 10 days post-transplant, osmotic pumps containing NSC23766 (2 pumps/mouse, 100 μM NSC23766 per pump) or PBS (as controls) were surgically implanted subcutaneously to allow continuous infusion of the inhibitor. After 14 days the pumps were replaced with fresh pumps and disease progression was monitored in mice exposed for up to 28 days. NSC23766-treated mice showed a significant increase in survival ($p < 0.01$) compared to control mice (**Figure 9A**). NSC23766 plasma levels were analyzed by HPLC and mass spectrometry and ranged from 1-3 μM (**Table 3**). This level of NSC23766 in control mice was associated with an expected doubling of the peripheral blood leukocyte count [40] (**Table 3**), providing pharmacodynamic evidence of the presence of the inhibitor. Finally, $CD34^+$ peripheral blood CML cells derived from leukapheresis products of two chronic-phase CML patients with significant leukocytosis (>300,000 WBC/mm^3) were transplanted into NOD/SCID mice and after engraftment for 10 days treated with NSC23766 by osmotic pumps for fourteen days. As shown in **Figure 9B**, compared to the PBS-treated control mice, NSC23766 induced ~85% reduction in human CML by 17 days post-transplant. Taken together, these data indicate that NSC23766 impairs p210-BCR-ABL-induced leukemogenesis in primary murine and human cells *in vivo*, and provides a rationale for targeting Rac proteins in this disease.

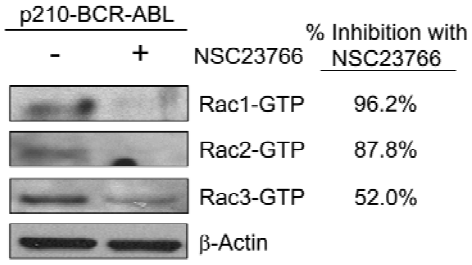

Figure 7. NSC23766 inhibits Rac1, Rac2 and Rac3 activation of p210-BCR-ABL-expressing wild-type BM cells. 5-FU-treated LDBM cells were transduced with p210-BCR-ABL, serum-starved overnight (16-18 hours) and treated with NSC23766 for 30 minutes. Cells were lysed and lysates submitted to PBD pull-down assays (Rac1-GTP, Rac2-GTP and Rac3-GTP) or loading control immunoblot (b-actin).

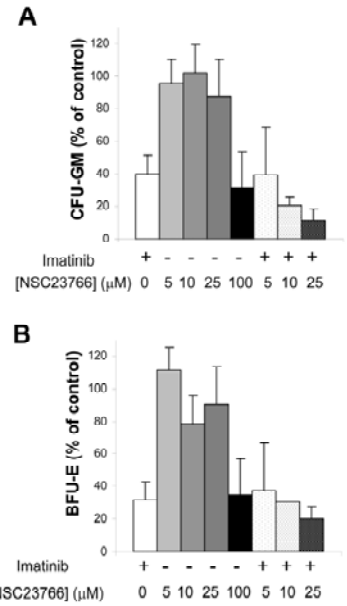

Figure 8. NSC23766 at 10-25 mM concentrations does not appear to significantly alter the proliferation of normal human bone marrow specimens, while 100 mM NSC23766 impairs human progenitor proliferation similar to 1 mM imatinib. Analysis of CFU-GM **(A)** or BFU-E **(B)** formation of normal human BM cells in the presence of imatinib (1 mM) and/or NSC23766 at different concentrations (n=5). Data represent mean ± SD of the level of inhibition reached for each specific specimen. * $p < 0.01$, ** $p < 0.001$.

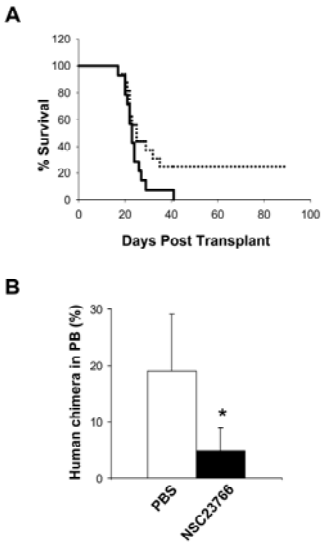

Figure 9. NSC23766 significantly delays the development of MSCV-p210-BCR-ABL-mediated leukemogenesis in a murine *in vivo* model and inhibits engraftment of human chronic phase CML cells in NOD/SCID mice. **A.** Survival curve of recipient mice that were transplanted with MSCV-p210-BCR-ABL-transduced wild type cells, then implanted with Alzet osmotic pumps containing either NSC23766 (2 pumps; 100 mM NSC23766 per pump; n=16) or PBS (1 pump; n=14) ten days post-transplant. Mice that died during surgery were censored from the study. * $p < 0.05$ (log P rank test) between PBS and NSC23766-treated groups. Data represent survival of pooled animals from two independent experiments with similar results. **B.** NOD/SCID mice were transplanted with CD34+ human CML peripheral blood cells from two newly diagnosed patients. Fifteen days post-transplant, Alzet osmotic pumps containing either NSC23766 (2 pumps, 75 mM NSC23766 per pump) or PBS (1 pump) were surgically implanted into the animals. Pre-implant levels of human chimera in peripheral blood were 66.3 ± 30.3% and 76.3 ± 9.9%, respectively. Seventeen days post-surgery, animals were sacrificed and human chimerism in peripheral blood was analyzed. Data represent mean ± SD of pooled data from two independent experiments (n=9 mice per group), * $p < 0.01$ (log P rank test).

Interestingly, the deficiency of Rac3 does not induce increased survival in this murine model. Our future studies intend to analyze the role of all Rac proteins in steady-state hematopoiesis in transgenic mice which inducibly express the p210-BCR-ABL gene under the control of the hematopoietic stem cell/progenitor transcriptional factor Scl promoter.

Table 3:

Pump	Plasma Concentration (μM)	WBC ($x10^3/mm^3$)
PBS	0.055	4.26
PBS	0.006	6.34
PBS	0.003	4.04
Average ± SD – PBS	**0.02 ± 0.03**	**4.88 ± 1.27**
NSC23766	3.1	6.32
NSC23766	0.95	10.5
NSC23766	1.09	4.88
Average ± SD - NSC23766	**1.71 ± 1.20**	**7.23 ± 2.92**

Key research accomplishments

1. Rac isoforms are overexpressed in human chronic-phase CML HSC/P.

2. A murine model of p210-BCR-ABL-induced MPD showed that gene targeting of *Rac1* and *Rac2* significantly delays or abrogates disease development.

3. Attenuation of the disease phenotype is associated with abolished p210-BCR-ABL-induced downstream signaling, including CrkL, Erk, p38, Jnk and Akt, and diminished STAT5 activation in primary hematopoietic cells along with compensatory hyperactivation of Rac3. Rac3 deficiency does not impair p210-BCR-ABL leukemogenesis.

4. A first generation, small molecule antagonist of Rac activation (NSC23766) validated biochemically and functionally Rac as a molecular target in both a relevant animal model and in primary human CML cells *in vitro* and in a xenograft model *in vivo*, including in Imatinib-resistant p210-BCR-ABL disease.

Reportable outcomes

Manuscripts:

1. Thomas EK*, Cancelas JA*, Zheng Yi, Williams DA. Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis. *Both authors contributed equally to this manuscript. Leukemia 2008 Epub Mar 20.
2. Williams DA, Zheng Y, Cancelas JA. Rho GTPases and regulation of hematopoietic stem cell localization. Methods Enzymol 2008;439:365-93.
3. Thomas EK*, Cancelas JA*, Chae H-D, Cox AD, Keller PJ, Perrotti D, Neviani, Druker BJ, Setchell KDR, Zheng Y, Harris CE, Williams DA. Rac guanosine triphosphatases are critical for the development of BCR/ABL-mediated chronic myeloid leukemia-like myeloproliferative disease. *Both authors contributed equally to this manuscript. Cancer Cell, 2007 Nov;12(5):467-78.

Abstracts

1. Sengupta A, **Cancelas** JA. Leukemic progenitor retention in the bone marrow niche is impaired in an inducible murine model of chronic myelogenous leukemia (CML). Submitted to the VI Annual Midwest Blood Club Meeting, Columbus, Ohio, April 3, 2008.
2. Sanchez-Aguilera A, Sengupta A, Williams DA, **Cancelas** JA. Role of Rac GTPase in p190 BCR-abl-induced B-cell acute lymphoblastic leukemia. Submitted to the VI Annual Midwest Blood Club Meeting, Columbus, Ohio, April 3, 2008.
3. Sanchez-Aguilera A, Sengupta A, Williams DA, **Cancelas** JA. Rac GTPases are activated in p190-BCR-ABL+ acute lymphoblastic leukemia. 10th Annual Ohio Comprehensive Cancer Center Scientific Meeting, Columbus OH, February 22, 2008.
4. Thomas EK, **Cancelas** JA, Chae H, Cox AD, Keller PJ, Perrotti D, Neviani P, Druker BJ, Zheng Y, Harris CE, Williams DA. Rac GTPases are potential therapeutic targets in p210-BCR-ABL-induced myeloproliferative disease (MPD). Oral Session, Amer Soc Hematol Meeting, Atlanta GA, Dec 8-11, 2007. Blood 2007;110(11);143a(465).
5. Sanchez-Aguilera A, **Cancelas** JA, Williams DA. RhoH-deficient mice show altered B cell populations in vivo. Poster, Amer Soc Hematol Meeting, Atlanta GA, Dec 8-11, 2007. Blood 2007;110(11);683a(2307).

Presentations

1. “Rac GTPases as molecular targets for BCR/ABL-induced leukemogenesis.” National Institute on Aging, NIH, Nov 16, 2007.
2. “Targeting Rac in chronic myelogenous leukemia.” Markey Cancer Center, University of Kentucky, Lexington, Aug 23, 2007.

Conclusion

We here have shown that Rac2, and especially the combination of Rac1 and Rac2 deficiency, induces increased survival in a murine model of CML induced by p210-BCR-ABL. We have also shown that this is due to a significant decrease of the downstream signaling depending upon p210-BCR-ABL. Pharmacollogical intervention through a drug which inhibits the activation of Rac GTPases impairs murine and human blastic-phase leukemic outgrowth in vitro and in a murine model and in a xenogeneic transplantation model of chronic-phase CML in vivo.

“So what?”

The results presented above indicate that targeting Rac GTPase represent a novel therapeutic approach in the therapy of chronic phase and blastic phase CML, which probably allow the management of tyrosine-kinase resistant, chronic-phase CML and blastic-phase CML patients.

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Appendices:

1. Thomas EK*, Cancelas JA*, Chae H-D, Cox AD, Keller PJ, Perrotti D, Neviani, Druker BJ, Setchell KDR, Zheng Y, Harris CE, Williams DA. Rac guanosine triphosphatases are critical for the development of BCR/ABL-mediated chronic myeloid leukemia-like myeloproliferative disease. Cancer Cell, 2007 Nov;12(5):467-78.
2. Supplementary data on the previous article.
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4. Thomas EK*, Cancelas JA*, Zheng Yi, Williams DA. Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis. *Both authors contributed equally to this manuscript. Leukemia 2008 Epub Mar 20.

Cancer Cell
Article

Rac Guanosine Triphosphatases Represent Integrating Molecular Therapeutic Targets for BCR-ABL-Induced Myeloproliferative Disease

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SUMMARY

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease (MPD) initiated by expression of the p210-BCR-ABL fusion protein. We demonstrate in a murine model of p210-BCR-ABL-induced MPD that gene targeting of *Rac1* and *Rac2* significantly delays or abrogates disease development. Attenuation of the disease phenotype is associated with severely diminished p210-BCR-ABL-induced downstream signaling in primary hematopoietic cells. We utilize NSC23766, a small molecule antagonist of Rac activation, to validate biochemically and functionally Rac as a molecular target in both a relevant animal model and in primary human CML cells in vitro and in a xenograft model in vivo, including in Imatinib-resistant p210-BCR-ABL disease. These data demonstrate that Rac is an additional therapeutic target in p210-BCR-ABL-mediated MPD.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease (MPD) initiated by malignant transformation of hematopoietic stem cells (HSC). The characterizing feature of this disease is the presence of the Philadelphia Chromosome [t(9;22)(q34;q11)], a somatic mutation in which the 3′ region of the Abelson leukemia virus (*ABL*) gene is fused to the 5′ region of the breakpoint cluster region (*BCR*) gene (Konopka et al., 1985; Shtivelman et al., 1985; Daley et al., 1990; Kelliher et al., 1990; Lugo et al., 1990). The p210 isoform of the resulting BCR-ABL fusion protein is necessary and sufficient for the development of CML (Daley et al., 1990). Expression of p210-BCR-ABL, a constitutively active tyrosine kinase, regulates a variety of signaling cascades, including Ras, extracellular-signal regulated kinase (ERK), Akt, c-Jun-activated kinase (JNK), p38, CrkL, signal transducer and activator of transcription 5 (STAT5), and nuclear factor-κB (NF-κB) (Ren, 2005); confers a proliferative advantage

SIGNIFICANCE

The introduction of tyrosine kinase inhibitors for the therapy of CML has extended the survival of CML patients by inducing long-term hematologic remissions. However, some proportion of CML patients demonstrate p210-BCR-ABL persistence at the molecular level and/or relapse with ABL kinase-inhibitor- resistant disease suggesting that inhibition of this kinase activity alone is not sufficient to eliminate all leukemic cells. Here, we show that the combined deficiency of Rac1 and Rac2 Rho GTPases significantly attenuates p210-BCR-ABL-induced proliferation in vitro and MPD in vivo and confirm Rac as a therapeutic target in p210-BCR-ABL disease using a first generation small molecule inhibitor.

to cells; and induces abnormal adhesion and migration of hematopoietic progenitor cells (Ramaraj et al., 2004; Zhao et al., 2001). p210-BCR-ABL expression appears to be directly responsible for the development of a transformed phenotype (Koschmieder et al., 2005).

Although allogeneic bone marrow (BM) transplantation is a curative treatment for CML, only ~25% of CML patients are eligible for this therapy (Goldman, 1997). Imatinib mesylate, an Abl kinase inhibitor, has been identified as an effective treatment option for p210-BCR-ABL-mediated leukemia (Druker et al., 1996). However, molecular remissions in response to Imatinib are not uniform and kinase domain mutations have been identified that are resistant to Imatinib therapy (Gorre et al., 2002; Lowenberg, 2003). Signaling proteins downstream of p210-BCR-ABL therefore may offer additional targets for treating p210-BCR-ABL-persistent and Imatinib-resistant disease.

The Rac subfamily of Rho guanosine triphosphatases (GTPases) plays an essential role in regulating hematopoiesis (Gu et al., 2003; Yang et al., 2001; Cancelas et al., 2005; Cancelas and Williams, 2006). Rho GTPases are Ras-like molecular switches that cycle between inactive, GDP-bound and active, GTP-bound states. In hematopoietic cells, Rac proteins integrate signals from growth factor, chemokine, and adhesion receptors to induce and coordinate a variety of cellular responses (Gu et al., 2003). The Rac subfamily is comprised of three highly homologous proteins: Rac1, Rac2, and Rac3. Rac2 is expressed specifically in hematopoietic cells, while Rac1 and Rac3 are ubiquitously expressed (Moll et al., 1991; Shirsat et al., 1990; Haataja et al., 1997). Although structurally similar, Rac1 and Rac2 share distinct as well as overlapping roles in the development and function of hematopoietic stem and progenitor cells (HSC/P). Rac1 is required for engraftment of HSC into the stem cell niche and regulates cell-cycle progression (Gu et al., 2003; Cancelas et al., 2005), whereas Rac2 is important for retention of HSC/P within the hematopoietic microenvironment (Yang et al., 2001) and regulates survival (Gu et al., 2003). Combinatorial expression of both proteins is necessary for normal HSC adhesion and migration and supports long-term hematopoiesis (Gu et al., 2003; Cancelas et al., 2005). Rac1 and Rac2 also regulate distinct aspects of cytoskeletal reorganization (Filippi et al., 2004). The role of Rac3 in hematopoiesis, which was initially cloned from a CML-derived cell line (Haataja et al., 1997), has not yet been fully defined.

Rac GTPases have been previously implicated in p210-BCR-ABL-mediated transformation (Sini et al., 2004; Renshaw et al., 1996; Skorski et al., 1998; Harnois et al., 2003; Burridge and Wennerberg, 2004; Schwartz, 2004), although the specific role(s) of individual Rac subfamily members in the development of disease in vivo have not been defined. Recent evidence also suggests that Rac3 plays a role in p190-BCR-ABL-mediated ALL, while Rac1 and Rac2 do not appear to be hyperactivated in these lymphoma lysates (Cho et al., 2005). This is of particular relevance, since p190-BCR-ABL differs from p210 in potentially important ways as it relates to RhoGTPases. For instance, while p210-BCR-ABL binds to and activates the Rho GTPases, apparently through the Dbl homology domain, p190-BCR-ABL, which lacks this domain, cannot bind to Rho GTPases but can still activate Rac1 and Cdc42 (Harnois et al., 2003) through activation of the guanine exchange factor (GEF) Vav1 by BCR-ABL (Bassermann et al., 2002). Rac GTPases have been shown to regulate signaling pathways that also are downstream of p210-BCR-ABL (Burridge and Wennerberg, 2004; Schwartz, 2004). Together, these data suggest that Rac GTPases may integrate multiple signaling components of p210-BCR-ABL-activated pathways.

We utilized a retroviral murine model in gene-targeted BM cells and analyzed primary human CML cells in vitro and in vivo in a xenograft model to investigate the importance of Rac GTPase activation in the development and progression of p210-BCR-ABL-mediated MPD. Here, we show that Rac GTPases are activated by p210-BCR-ABL, and the combined deficiency of Rac1 and Rac2 significantly attenuates p210-BCR-ABL-induced proliferation in vitro and MPD in vivo. Attenuation of the disease phenotype is associated with severely diminished p210-BCR-ABL-induced downstream signaling in primary hematopoietic cells. We utilize NSC23766, a small molecule antagonist of Rac activation, to biochemically and functionally validate Rac as a molecular target in both a relevant animal model and in primary human CML cells in vitro and in a xenograft model in vivo, including in Imatinib-resistant p210-BCR-ABL disease. These data demonstrate that Rac is an additional therapeutic target in p210-BCR-ABL-mediated MPD.

RESULTS

Rac Is Hyperactivated in Chronic-Phase CML HSC/P

Recent studies in cell lines have suggested that Rho GTPases can be activated by p210-BCR-ABL in vitro and in vivo (Skorski et al., 1998; Harnois et al., 2003). Since the expression of p210-BCR-ABL in HSC appears to be sufficient to induce a transformation phenotype, we first analyzed whether Rac isoforms were hyperactivated in human chronic phase CML HSC/P. Activation of Rac was determined by p21-activated kinase (PAK)-binding domain (PBD) pull-down assays in isolated CD34+ cells from CML patients. We observed that Rac1, Rac2, and, to a lesser degree, Rac3 were hyperactivated in $CD34^+$ cells purified from peripheral blood of two CML patients at diagnosis (Figure 1A). To determine the effect of p210-BCR-ABL expression on activation of the Rac subfamily of Rho GTPases in a murine model of p210-BCR-ABL disease, we exogenously expressed p210-BCR-ABL in primary murine HSC/P. 5-fluorouracil (5-FU)-treated low-density BM (LDBM) cells were transduced with bicistronic vectors expressing enhanced green fluorescent protein (EGFP) either alone (empty vector, MIEG3) or with p210-BCR-ABL (MSCV-p210-BCR-ABL) (Hawley et al., 1993). Sorted, $EGFP^+$ cells were starved and then stimulated with stromal-derived factor-1α (SDF-1α), a chemokine

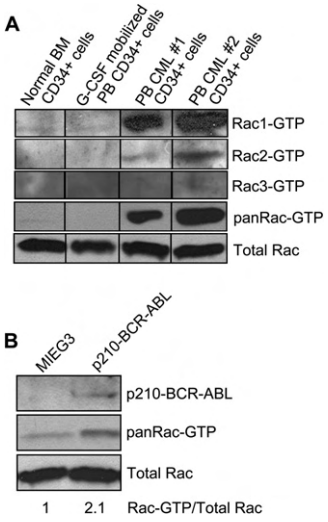

Figure 1. Rac GTPases Are Hyperactivated in Chronic-Phase Human CML HSC/P and Murine HSC/P Expressing p210-BCR-ABL

(A) Primary human CD34+ BM or G-CSF-mobilized peripheral blood cells and CD34+ chronic-phase CML peripheral blood cells (from two different CML patients) were starved for 1 hr and analyzed for Rac activation in a PAK-binding domain (PBD) pull-down assay. Samples were blotted and analyzed for Rac1-GTP, Rac2-GTP, Rac3-GTP, total Rac-GTP, and total Rac protein content.

(B) 5-FU-treated murine LDBM cells were transduced with either MIEG3 or MSCV-p210-BCR-ABL, bicistronic vectors expressing EGFP. The EGFP$^+$ cells were sorted, serum-starved for 6 hours, stimulated with 100 ng/ml SDF-1 for 5 minutes, lysed, and used in a PAK-binding domain (PBD) pull-down assay. The ratio of GTP-bound Rac to total Rac was determined by densitometry. The data represent one of three experiments with similar results.

known to induce migration of p210-BCR-ABL-expressing HSC/P (Zhao et al., 2001). Expression of p210-BCR-ABL in LDBM cells, confirmed by immunoblot, led to a >2-fold increase in GTP-bound Rac (Figure 1B) compared to MIEG3 transduced cells.

Rac1 and Rac2 Deficiency Significantly Attenuates p210-BCR-ABL-Mediated MPD In Vivo

To determine whether and which Rac GTPases are required for the development of p210-BCR-ABL-induced leukemogenesis in vivo, we utilized gene-targeted mice deficient in *Rac2* and with conditional (floxed) alleles of *Rac1* in a retroviral murine model of CML. 5-FU-treated *Cre^{Tg+};WT*, *Cre^{Tg+};Rac1$^{flox/flox}$*, *Cre^{Tg+};Rac2$^{-/-}$*, and *Cre^{Tg+};Rac1$^{flox/flox}$;Rac2$^{-/-}$* LDBM cells were transduced with MIEG3 (control, empty vector) or MSCV-p210-BCR-ABL and sorted for EGFP$^+$ expression. Irradiated C57Bl/6 mice were transplanted with 50,000–75,000 of the EGFP$^+$ transduced cells together with 500,000 unmanipulated BM cells to assure the rescue of normal hematopoiesis in the postirradiation period. Ten days posttransplant, recipient mice were treated with polyI:C as previously described (Gu et al., 2003; Cancelas et al., 2005) to delete floxed *Rac1* genomic sequences (hereafter designated *Rac1$^{\Delta/\Delta}$* mice). Recipient mice transplanted with MSCV-p210-BCR-ABL-transduced *Cre^{Tg+};WT* or *Cre^{Tg+};Rac1$^{flox/flox}$* LDBM cells that were treated with PolyI:C uniformly developed CML-like MPD (leukocytosis, splenomegaly, pulmonary hemorrhage, and extensive liver infiltration with hematopoietic cells at necropsy) and died within forty days posttransplant (Figure 2A), consistent with the MPD phenotype, while all of the mice transplanted with MIEG3-transduced WT or *Rac1$^{\Delta/\Delta}$* cells were still alive at day 100 posttransplant (Table S1). Strikingly, mice transplanted with p210-BCR-ABL-expressing *Cre^{Tg+};Rac1$^{flox/flox}$;Rac2$^{-/-}$* cells that were treated with polyI:C to delete *Rac1* in the *Rac2* null background (designated *Rac1$^{\Delta/\Delta}$;Rac2$^{-/-}$* mice) showed significantly prolonged survival (Figure 2A; $p < 0.001$). Nearly 50% of these mice were still alive 100 days posttransplant. PCR analysis confirmed loss of exon 1 of *Rac1* in *Rac1$^{\Delta/\Delta}$* and *Rac1$^{\Delta/\Delta}$;Rac2$^{-/-}$* mice treated with polyI:C (Figure 2B). Clonal analysis by linear amplification-mediated polymerase chain reaction (LAM-PCR) of BM cells from leukemic mice showed similar and oligoclonal reconstitution of p210-BCR-ABL-expressing wild-type, *Rac1$^{\Delta/\Delta}$*, *Rac2$^{-/-}$* and *Rac1$^{\Delta/\Delta}$;Rac2$^{-/-}$* cells (Figure 2C), suggesting that the delay in disease progression in these animals was not due to loss of p210-BCR-ABL vector integration and expression. Southern blot analysis confirmed the LAM-PCR study, showing 1–3 major clones/leukemia and no differences in the number of clones between genotypes (data not shown). All recipient mice maintained peripheral EGFP$^+$ cells throughout the study, confirming sustained engraftment of p210-BCR-ABL-expressing cells even in the absence of Rac1 and Rac2 (Figure 2D), a finding that is noteworthy due to our previous observations that hematopoietic engraftment of *Rac1$^{\Delta/\Delta}$;Rac2$^{-/-}$* HSC/Ps is rapidly lost in the absence of p210-BCR-ABL (Cancelas et al., 2005). Survival of mice transplanted with p210-BCR-ABL-transduced *Rac2$^{-/-}$* cells was intermediate to *Rac1$^{\Delta/\Delta}$;Rac2$^{-/-}$* mice and significantly longer ($p < 0.001$) than the WT and *Rac1$^{\Delta/\Delta}$* mice (Figure 2A).

We next confirmed that the increased survival seen in Rac-deficient, p210-BCR-ABL-expressing mice was not related to defective engraftment of Rac-deficient HSC. To analyze the homing and engraftment of p210-BCR-ABL-expressing cells in the presence or absence of the Rac GTPases, recipient mice were transplanted with PKH26-labeled WT and *Rac1$^{flox/flox}$;Rac2$^{-/-}$* donor BM cells transduced with p210-BCR-ABL. The percentages of PKH26/EGFP-expressing cells in BM at 16 hr post-transplantation were similar between the WT and *Rac1$^{flox/flox}$;Rac2$^{-/-}$* animals, suggesting unimpaired homing despite lack of Rac2 expression (Figure S1A), consistent with our previous findings in normal hematopoietic cells (Cancelas et al., 2005). In addition, there was an equivalent frequency of EGFP$^+$Lin$^-$Sca1$^+$c-Kit$^+$ cells observed in the BM of recipient mice transplanted with either WT or *Rac1$^{flox/flox}$;Rac2$^{-/-}$* p210-BCR-ABL-expressing cells 18 days posttransplant after deletion of Rac1 sequences, suggesting that Rac-deficient HSC/P maintain an early graft as efficiently as wild-type cells in

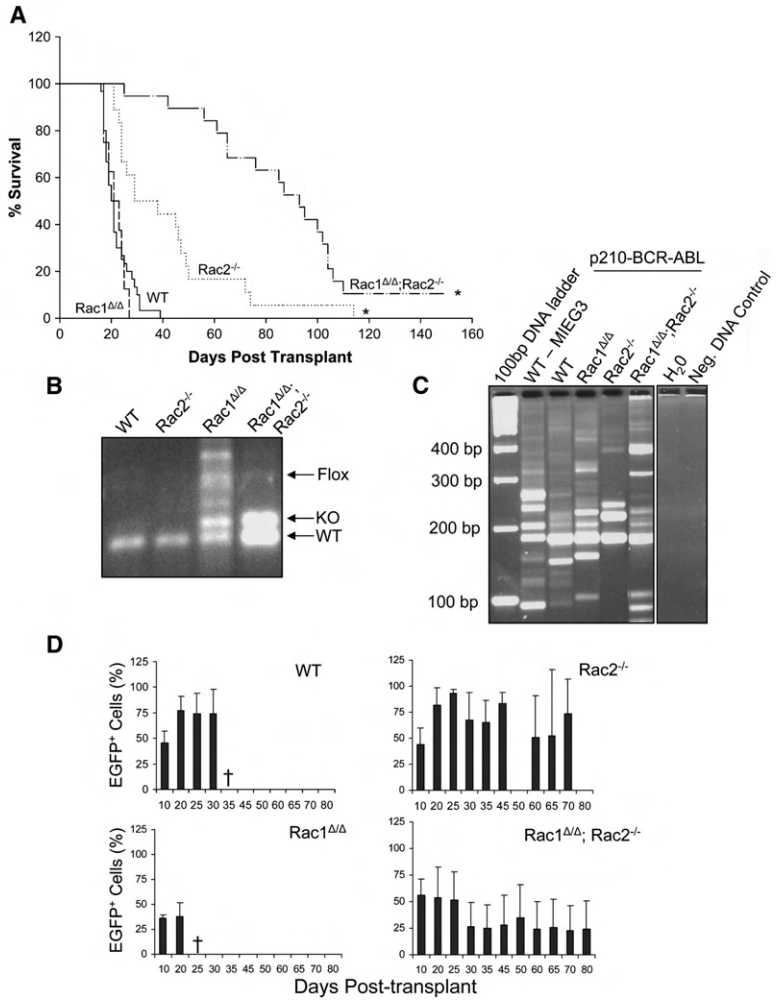

Figure 2. Rac GTPases Are Critical Regulators of p210-BCR-ABL-Mediated Leukemogenesis

(A) Kaplan-Meier survival curve of mice that were transplanted with MSCV-p210-BCR-ABL-transduced wild-type and Rac-deficient cells. Mice demonstrating engraftment (monitored by the percentage of EGFP+ cells in the peripheral blood) of less than 1% at two consecutive time points were censored from the study. Genotypes are abbreviated in all figures as follows: WT, wild-type or flox allele at *Rac1* and *Rac2* loci, n = 30; $Rac2^{-/-}$, wild-type or flox allele at *Rac1* and null allele at *Rac2* locus, n = 18; $Rac1^{\Delta/\Delta}$, Cre-mediated null allele at *Rac1* locus and wild-type allele at Rac2 locus, n = 8; $Rac1^{\Delta/\Delta};Rac2^{-/-}$, Cre-mediated null allele at *Rac1* locus and null allele at *Rac2* locus, n = 19. *p < 0.001 (log P rank test) between MSCV-p210-BCR-ABL-expressing WT and $Rac1^{\Delta/\Delta}$ recipient mice and either BCR-ABL-transduced $Rac2^{-/-}$ or $Rac1^{\Delta/\Delta};Rac2^{-/-}$ groups.

(B) Representative PCR showing deletion of the Rac1 floxed gene in the peripheral blood of $Rac1^{\Delta/\Delta}$ and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ recipient mice 70 days posttransplant, as visualized by the presence of the knockout (KO) band in the representative $Rac1^{\Delta/\Delta}$ and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ animals. The presence of the wild-type (WT) allele in these mice does not signify expression of the $Rac1^{flox/flox}$ allele but rather represents contribution from unmanipulated BM cells coinjected with BCR-ABL-transduced cells. Thus, the efficiency of loss of exon 1 of *Rac1* can be determined by comparing the Flox band and the KO band in these samples.

(C) LAM-PCR amplifying retroviral vector insertion sites in p210-BCR-ABL-expressing BM cells from mice reconstituted with wild-type, $Rac1^{\Delta/\Delta}$, $Rac2^{-/-}$ and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ cells. 3/3 leukemic $Rac1^{\Delta/\Delta};Rac2^{-/-}$ animals tested demonstrated oligoclonal integration patterns.

(D) Percentage of $EGFP^+$ cells in the peripheral blood of all surviving mice monitored over the course of the transplant. Data represent mean ± SD of all the mice included in Figure 2A.

the presence of p210-BCR-ABL (Figure S1B). In agreement with these data, there was no significant difference in expression of the hyaluronan receptor, CD44, which has recently been shown to play a specific and essential role in the homing and engraftment of p210-BCR-ABL-expressing leukemia-initiating cells (Krause et al., 2006) either in vitro (Figure S1C), using cotransduced (Cre-YFP and p210-BCR-ABL-EGFP) and sorted $Rac1^{\Delta/\Delta};Rac2^{-/-}$ LDBM cells, or in vivo (Figure S1D), from animals injected with p210-BCR-ABL-expressing $Rac1^{flox/flox};Rac2^{-/-}$ cells, compared to p210-BCR-ABL-expressing wild-type cells. Thus, these data suggest that prolonged survival of mice transplanted with p210-BCR-ABL-transduced Rac-deficient cells is not explained by reduced engraftment of leukemia-initiating stem cells.

Phenotype of MPD in the Rac-Deficient Mice

p210-BCR-ABL-expressing WT mice rapidly developed significant EGFP+ leukocytosis that persisted until death (Figures 2D and 3A). p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta}$ mice succumbed to splenomegaly and pulmonary hemorrhage (Table S1), consistent with the MPD phenotype. The majority of p210-BCR-ABL $Rac2^{-/-}$ mice showed gradual progression of leukocytosis and eventually died of MPD (Figure 3A; Table S2). Differential counts of the peripheral blood from p210-BCR-ABL-expressing WT and $Rac2^{-/-}$ mice ~30 days posttransplant demonstrated neutrophilia and the presence of immature granulocyte precursors and blasts in the peripheral blood (Figures 3B and 3C), consistent with the MPD previously described in this model (Daley et al., 1990; Kelliher et al., 1990). As expected, development of leukemia in these mice was accompanied by a predominance of $EGFP^+$ cells in the blood (Figure 2D), BM, and spleen (data not shown). $EGFP^+$ cells in the spleen and BM of all p210-BCR-ABL-transplanted WT, $Rac1^{\Delta/\Delta}$, and $Rac2^{-/-}$ recipient mice were uniformly $Gr\text{-}1^+/Mac\text{-}1^+$ (Table S2). In contrast to these mice, p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta};Rac2^{-/-}$

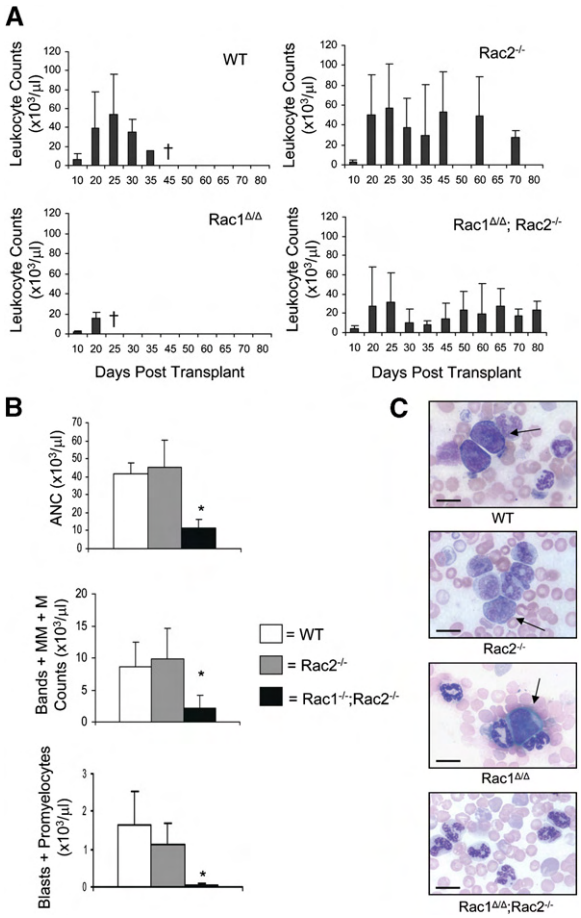

Figure 3. Deficiency of Rac1 and Rac2 Expression Significantly Delays or Appreciably Inhibits the Development of Leukocytosis in BCR-ABL-Expressing Recipient Mice

(A) Time course showing average leukocyte counts in the peripheral blood of recipient mice that were injected with either WT or Rac-deficient LDBM cells transduced with MSCV-p210-BCR-ABL. WT, n = 30; *Rac2*$^{-/-}$, n = 18; *Rac1*$^{\Delta/\Delta}$, n = 8; *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ n = 19. Data represent mean ± SD. Crosses represent time points at which no animals were surviving for analysis.

(B) Differential counts show decreased frequency of blasts and other immature myeloid progenitors in the peripheral blood of *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ BCR-ABL-transduced recipient mice approximately 30 days post-transplant, compared to the WT and *Rac2*$^{-/-}$ mice. Data represent mean ± SD. ANC, average neutrophil counts; MM, metamyelocytes; M, myelocytes. WT, n = 7; *Rac2*$^{-/-}$, n = 7; *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$, n = 8. *p < 0.05 between BCR-ABL-expressing WT and *Rac2*$^{-/-}$ mice and the BCR-ABL-transduced *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ group.

(C) Morphology of cells present in peripheral blood smears from representative BCR-ABL-transduced WT and Rac-deficient recipient mice approximately 30 days posttransplant. Myeloblasts (arrows) were apparent in all mice except for BCR-ABL-recipient *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice. Bars, 10 μm.

recipient mice showed normal peripheral blood morphology at ~30 days posttransplant in spite of significant chimerism with EGFP^{+} p210-BCR-ABL-expressing cells (Figures 2D, 3B, and 3C). The few p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice that developed early disease (arbitrarily defined as ≤69 days; 4/19 *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ mice) had either a myeloid (Gr-1^{+}/Mac-1^{+}, 67% of three animals tested) or lymphoid (B220^{+}, 33% of three animals tested) phenotype. One of the mice with a myeloid phenotype developed a solid tumor in the spine that showed high (86%) EGFP expression. The mice that succumbed to late disease (≥70 days; 15/19 *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ mice) had either a myeloid (Gr-1^{+}/Mac-1^{+}, 62% of 13 animals tested), lymphoid (B220^{+}, 23% of 13 animals tested), or bilineage (myeloid and lymphoid; 15% of 13 animals tested) phenotype. Additionally, two of the p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice with late onset disease developed solid tumors in the skull and brain, with histochemical and histological features consistent with CD68^{+} histiocytic sarcoma. Two of the p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice developed solid tumors in the spine with high (>70%) EGFP expression. One of the animals with a high percentage (40%) of CD3^{+} cells also presented with a tumor on the left kidney.

Activation of Signaling Cascades in Transformed Cells of p210-BCR-ABL-Expressing Mice

These data show that loss of Rac1 and Rac2 expression plays a key role in attenuation of the MPD phenotype, but suggest that late molecular events may overcome the loss of Rac1 and Rac2 function. To assess the status of Rac activation in p210-BCR-ABL-expressing WT, *Rac1*$^{\Delta/\Delta}$, *Rac2*$^{-/-}$, and *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ mice developing MPD, we performed PBD pull-down assays on splenocytes of diseased animals. Although *Rac1* and *Rac2* gene deletion was confirmed by PCR analysis (data not shown), active GTP-bound Rac as detected by a pan-Rac antibody was elevated in all p210-BCR-ABL-expressing leukemic mice tested (Figure 4A), suggesting that the third member of the Rac subfamily, Rac3, may be activated in p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ leukemic mice. Rac3 expression was confirmed in all leukemic animals tested by immunoblot (Figure 4B, lower panel) and PBD pull-down assays of splenocytes harvested from additional diseased animals showed enhanced GTP-bound Rac3 in p210-BCR-ABL-expressing leukemic mice, most clearly in the *Rac2*$^{-/-}$ and *Rac1*$^{\Delta/\Delta}$*; Rac2*$^{-/-}$ samples (Figure 4B, upper panel), suggesting that Rac3 likely plays a key role in the eventual development of CML-like MPD.

Activation of Rac by p210-BCR-ABL in the WT mice was associated with increased baseline ERK, JNK, p38, Akt, STAT5, and CrkL phosphorylation (Figure 4C). These results are consistent with Rac signaling pathways previously implicated by us and others (Ren, 2005; Gu et al., 2003). Activation of ERK, JNK, p38, and CrkL was similar to WT mice in *Rac1*$^{\Delta/\Delta}$ mice, but was reduced in *Rac2*$^{-/-}$ and nearly completely abrogated in *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ splenocytes harvested from mice that developed MPD, despite continued activation of BCR-ABL in p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$*;Rac2*$^{-/-}$ splenocytes as determined by phospho-tyrosine immunoblots and increased activation of Rac3 (Figures 4B and 4D). These biochemical findings are strikingly in parallel with the survival curves of p210-BCR-ABL-expressing mice. Phosphorylation of Akt

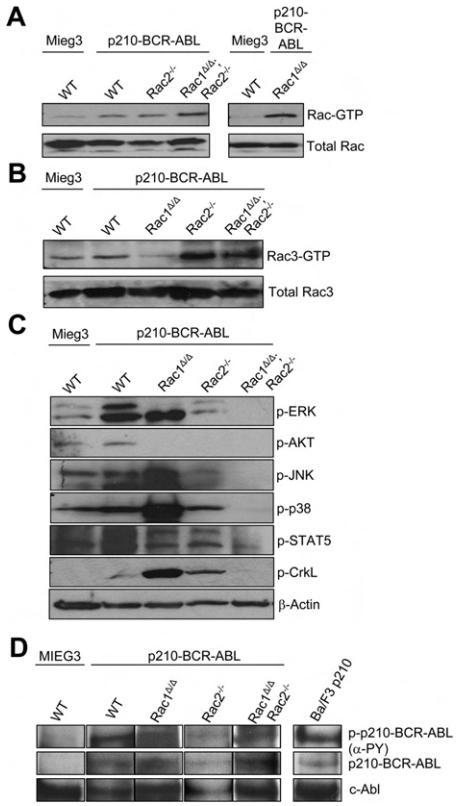

Figure 4. Rac GTPases Are Implicated in BCR-ABL-Mediated Activation of Multiple Signaling Cascades

(A) Representative example of Rac activation (PBD) pull-down assays performed on splenocytes harvested from MIEG3 and MSCV-p210-BCR-ABL-transduced WT and Rac-deficient mice developing MPD. Top panel (Rac-GTP) represents activated total Rac; lower panel represents total Rac protein expressed by immunoblot.

(B) Representative example of PBD pull-down assays of splenocytes harvested from leukemic animals to monitor Rac3 activation, using a Rac3-specific antibody.

(C) Representative examples of immunoblot analyses of splenocytes from BCR-ABL-expressing WT and Rac-deficient recipient mice developing MPD using phospho-antibodies specific to ERK, JNK, p38, Akt, CrkL, and STAT5. β-actin was used as a loading control. For each analysis, a minimum of three specimens from different mice were analyzed with similar results.

(D) Activation of p210-BCR-ABL in BM cells harvested from deceased or sacrificed leukemic mice. Phospho-p210-BCR-ABL expression is demonstrated in leukemic WT, *Rac1*$^{\Delta/\Delta}$, *Rac2*$^{-/-}$, and *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BM cells with a phospho-tyrosine antibody. A minimum of 3 samples in each genotype confirmed phosphorylation of p210-BCR-ABL in these samples. Expression of total p210-BCR-ABL and c-Abl was visualized using a c-Abl antibody. As a positive control, lysates from Ba/F3 cells stably expressing p210-BCR-ABL were analyzed.

was reduced in all of the Rac-deficient mice compared with WT mice. These data strongly suggest that activation of multiple signaling pathways by p210-BCR-ABL is dependent on Rac1 and Rac2. Interestingly, STAT5 phosphorylation was variably diminished in leukemic splenocytes even in the absence of Rac2 and more severely reduced but still detectable in the absence of both Rac1 and Rac2. p210-BCR-ABL, thus, may mediate activation of this pathway via induced Rac3, and/or STAT5 may be activated independently of Rac GTPases.

Rac Is a Molecular Target in p210-BCR-ABL-Expressing Cells

These results strongly support the hypothesis that p210-BCR-ABL signaling is dependent on Rac activation, suggesting that Rac GTPases may be unique molecular targets for CML therapy. We next used a genetic approach to determine whether deficiency of Rac1 and Rac2 influences p210-BCR-ABL-induced hyperproliferation of hematopoietic cells in vitro, a characteristic of this retroviral model of p210-BCR-ABL expression. LDBM cells were harvested from *Rac1*$^{flox/flox}$ and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ mice and cotransduced with MSCV-Cre-YFP and either MIEG3 or MSCV-p210-BCR-ABL. Deletion of *Rac1* in the Cre-YFP-expressing cells was confirmed by PCR analysis (data not shown). Proliferation of sorted EGFP$^+$ (*Rac1*$^{flox/flox}$) and EGFP$^+$/YFP$^+$ (*Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$) cells was determined by thymidine incorporation and cell counts. As shown in Figure 5A, p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$ primary hematopoietic cells displayed significantly increased proliferation compared to MIEG3-transduced *Rac1*$^{flox/flox}$ cells. *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ cells exhibited significantly reduced p210-BCR-ABL-mediated hematopoietic cell proliferation, compared to p210-BCR-ABL-expressing *Rac1*$^{flox/flox}$ cells, suggesting that p210-BCR-ABL-mediated hyperproliferation of hematopoietic cells in vitro is dependent on activation of Rac1 and Rac2 and further validating Rac GTPases as key regulators of p210-BCR-ABL-mediated MPD.

To examine if pharmacologic inhibition of Rac in the presence of p210-BCR-ABL leads to attenuated cell proliferation, WT LDBM cells transduced with either MSCV-p210-BCR-ABL or MIEG3 and sorted for EGFP$^+$ expression were serum starved and incubated for 48 hr in the presence of increasing concentrations of NSC23766, a Rac-specific small molecule inhibitor that has been shown to inhibit Rac1 and Rac2 (Gu et al., 2003; Cancelas et al., 2005) and that we have found to inhibit Rac1, Rac2, and Rac3 in BCR-ABL-transduced LDBM cells (see Figure S2). NSC23766 does not inhibit RhoA or Cdc42 (Gao et al., 2004). Although inhibition of Rac activation with NSC23766 is reversible and less complete than genetic deletion, p210-BCR-ABL-induced proliferation was inhibited in a dose-dependent manner (Figure 5B, solid bars). No inhibition of MIEG3-transduced cells was detected at the same concentrations of inhibitor (Figure 5B, empty bars). Since we hypothesized that Rac3 was abnormally activated in splenocytes harvested from p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice developing late MPD, the effect of the Rac-specific inhibitor NSC23766 was tested in vitro on BM cells derived from these mice. The proliferation of EGFP$^+$ *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ BM cells harvested from Rac1;Rac2-deficient mice with MPD was inhibited in a dose-dependent manner by the Rac inhibitor (Figure 5C), and immunoblotting confirmed

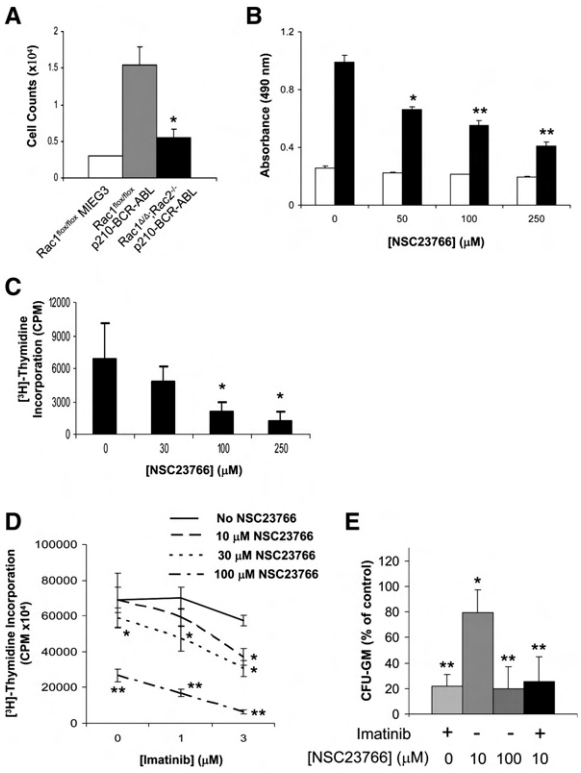

Figure 5. Loss of Rac Activation via Gene Deletion or by Treatment with NSC23766, a Rac-Specific Inhibitor, Diminishes the Proliferation of BCR-ABL-Expressing Murine and Human Cells

(A) 5-FU-treated *Rac1*$^{flox/flox}$ and *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ HSC were transduced with MSCV-Cre-YFP to delete the *Rac1* floxed genomic sequence, together with MIEG3 or MSCV-p210-BCR-ABL. The EGFP^{+} and EGFP^{+}/YFP^{+} cells were sorted and plated. Cell proliferation was determined by cell counts 48 hr after the cells were plated. Data represent mean ± SD, n = 3 in each of two independent experiments. *p < 0.05 between the BCR-ABL-transduced *Rac1*$^{flox/flox}$ cells and the MIEG3-expressing *Rac1*$^{flox/flox}$ and BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ cells.

(B) 5-FU treated WT murine LDBM cells were transduced with either MIEG3 (white bars) or MSCV-p210-BCR-ABL (black bars), and the EGFP^{+} cells were sorted. Cells were then plated in the presence of increasing concentrations of NSC23766, and proliferation was determined 48 hr later by MTS assay. Data represent mean ± SD, n = 3 for three independent experiments. *p < 0.01, **p < 0.001 versus no drug.

(C) Effect of NSC23766 on BM cells harvested from BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice that developed MPD. Results are representative of mean ± SD from proliferation assays performed on three different *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice in triplicate. *p < 0.05 versus no drug.

(D) Effect of increasing concentrations of NSC23766 alone and in combination with Imatinib on the proliferation of Imatinib-resistant Ba/F3 p210-BCR-ABL-T315I cells. Data represent mean ± SD of four independent assays per sample done in duplicate. *p < 0.01; **p < 0.001.

(E) Analysis of CFU-GM formation from CML patients that developed myeloid blast crises in the presence of 1 μM Imatinib (n = 10 patients), NSC23766 (n = 10 patients), or a combination of Imatinib and NSC23766 (n = 5 patients). Data represent mean ± SD of the level of inhibition reached for each specific specimen. *p < 0.01; **p < 0.001.

inhibition of Rac3 activation in leukemic samples harvested from *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ animals (data not shown). These results further implicate Rac3 in the late development of MPD in the *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice.

To determine the effect of Rac inhibition on proliferation of an Imatinib-resistant p210-BCR-ABL mutant (La Rosee et al., 2002, 2004), Ba/F3 cells expressing the highly resistant T315I-p210-BCR-ABL mutant were incubated with Imatinib and/or NSC23766. As reported previously (Corbin et al., 2003), addition of up to 3 μM Imatinib alone had little effect on proliferation of these cells (less than 10% inhibition, Figure 5D). However, T315I-expressing cells treated with NSC23766 alone or in combination with Imatinib showed >90% inhibition of proliferation, similar to Ba/F3 cells expressing nonmutated p210-BCR-ABL (Figure 5D). Finally, the effect of NSC23766 on the growth of primary BM cells from CML patients in blast crisis was evaluated. Addition of 10–100 μM NSC23766 inhibited 20%–78% of blastic phase CML BM colony forming unit-granulocyte/macrophage (CFU-GM) colonies, a response similar to 1 μM Imatinib (Figure 5E). Similar inhibition was observed for CML blast phase erythroid progenitor cells (data not shown), while 100 μM NSC23766, but not lower doses, inhibited normal human progenitor BM cells in a fashion similar to 1 μM Imatinib (Figures S3A and S3B). These data demonstrate that Rac is essential for p210-BCR-ABL-induced proliferation of primary cells, and pharmacologic inhibition of Rac significantly reduces p210-BCR-ABL-mediated proliferation even in the presence of highly resistant kinase mutants.

To determine the effect of NSC23766 in vivo, mice were transplanted with p210-BCR-ABL-expressing murine HSC/P as described above and 10 days posttransplant, osmotic pumps containing NSC23766 (2 pumps/mouse, 100 μM NSC23766 per pump) or PBS (as controls) were surgically implanted subcutaneously to allow continuous infusion of the inhibitor. After 14 days, the pumps were replaced with fresh pumps and disease progression was monitored in mice exposed for up to 28 days. NSC23766-treated mice showed a significant increase in survival ($p < 0.01$) compared to control mice (Figure 6A). NSC23766 plasma levels were analyzed by HPLC and mass spectrometry and ranged from 1–3 μM (Table S3). This level of NSC23766 in control mice was associated with an expected doubling of the peripheral blood leukocyte count (Roberts et al., 1999) (Table S3), providing pharmacodynamic evidence of the presence of the inhibitor. Finally, CD34^{+} peripheral blood CML cells derived from leukapheresis products of two chronic-phase CML patients with significant leukocytosis (>300,000 WBC/mm^3) were transplanted into NOD/SCID mice and, after engraftment for 10 days, treated with NSC23766 by osmotic pumps for 14 days. As shown in Figure 6B, compared to the PBS-treated control mice, NSC23766 induced ~85% reduction in human CML by 17 days posttransplant. Taken together, these data indicate that NSC23766 impairs p210-BCR-ABL-induced leukemogenesis in primary murine and human cells in vivo and provides a rationale for targeting Rac proteins in this disease.

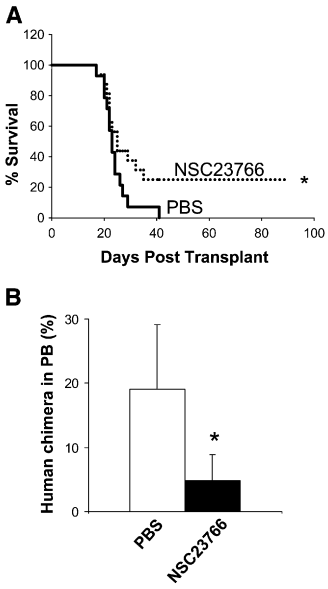

Figure 6. NSC23766 Significantly Delays the Development of MSCV-p210-BCR-ABL-Mediated Leukemogenesis in a Murine In Vivo Model and Inhibits Engraftment of Human Chronic Phase CML Cells in NOD/SCID Mice

(A) Survival curve of recipient mice that were transplanted with MSCV-p210-BCR-ABL-transduced wild-type cells, then implanted with Alzet osmotic pumps containing either NSC23766 (2 pumps; 100 mM NSC23766 per pump; n = 16) or PBS (1 pump; n = 14) ten days post-transplant. Mice that died during surgery were censored from the study. *$p < 0.05$ (log P rank test) between PBS and NSC23766-treated groups. Data represent survival of pooled animals from two independent experiments with similar results.

(B) NOD/SCID mice were transplanted with CD34+ human CML peripheral blood cells from two newly diagnosed patients. Fifteen days posttransplant, Alzet osmotic pumps containing either NSC23766 (2 pumps, 75 mM NSC23766 per pump) or PBS (1 pump) were surgically implanted into the animals. Preimplant levels of human chimera in peripheral blood were 66.3 ± 30.3% and 76.3 ± 9.9%, respectively. Seventeen days postsurgery, animals were sacrificed and human chimerism in peripheral blood was analyzed. Data represent mean ± SD of pooled data from two independent experiments (n = 9 mice per group), *$p < 0.01$ (log P rank test).

DISCUSSION

Imatinib (Gleevec, also known as STI571 or CGP57148), an Abl kinase inhibitor that shows significant activity in all phases of CML and Ph-positive acute leukemias (Druker et al., 2002) by selective induction of apoptosis of p210-BCR-ABL-positive cells (Druker et al., 1996; Deininger et al., 1997; le Coutre et al., 1999), has provided an effective means of treatment in CML. The persistence of p210-BCR-ABL-positive HSC in Imatinib-treated patients suggests that inhibition of Abl kinase activity alone might not be sufficient to eliminate all leukemic stem cells.

We have previously shown that both Rac1 and Rac2 are essential for the regulation of multiple HSC functions with unique as well as overlapping roles, including proliferation, apoptosis, homing, and retention (Gu et al., 2003; Cancelas et al., 2005). These biological functions of Rac are associated with activation of multiple kinase signaling cascades, many of which are activated in CML blasts or in cell lines expressing p210-BCR-ABL. Thus, Rac proteins may integrate signals affecting survival/proliferation and cytoskeletal rearrangements leading to the motility and adhesion phenotypes reported in these cells. These observations led us to examine the requirement of Rac proteins in p210-BCR-ABL-mediated transformation.

In the studies reported here, significantly prolonged survival in vivo of p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice is apparent despite the observation by LAM-PCR and Southern blotting that all genotypes demonstrate oligoclonality. One to three retroviral integrations of the p210-BCR-ABL vector were observed in each genotype by Southern blot, a result not surprising based on the number of transduced cells injected into each animal and assuming the development of a HSC-initiated disease.

The increased survival of p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ mice correlates with nearly complete elimination of baseline hyperactivation of ERK, p38, JNK, Akt, and CrkL in BM cells from these mice. BCR-ABL *Rac1*$^{\Delta/\Delta}$/*Rac2*$^{-/-}$ mice that did develop MPD invariably appeared to induce Rac3 activation. The data supporting this conclusion include the absence of Rac1 and Rac2 genomic sequences in the EGFP^{+} peripheral blood cells of diseased animals, the increased Rac3 activity in splenocytes from these animals shown by pull-down assays, and the inhibition of proliferation in vitro of BM cells from *Rac1*$^{\Delta/\Delta}$;*Rac2*$^{-/-}$ leukemic mice when incubated with NSC23766, a Rac-specific small molecule inhibitor (Gao et al., 2004). These data are consistent with previous reports of Rac3 activation in p190-BCR-ABL expressing malignant precursor B-lineage lymphoid cells (Cho et al., 2005).

p210-BCR-ABL-expressing *Rac2*$^{-/-}$ animals showed less reduction in the baseline activation of these pathways and less, although significant, prolongation of survival. p210-BCR-ABL-expressing *Rac1*$^{\Delta/\Delta}$ mice behaved similarly to WT mice in this model. These data strongly suggest that each Rac protein plays a specific role in p210-BCR-ABL-mediated leukemogenesis, as we have noted in normal HSC/P functions (Gu et al., 2003; Cancelas et al., 2005). Alternately, these phenotypes could represent a combinatorial but variable decrease in total Rac activity. Studies are underway to determine the individual and combinatorial role(s) of each Rac protein, including Rac3, using *Rac3*$^{-/-}$ mice (Corbetta et al., 2005) bred into the *Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ mouse line.

CrkL has been suggested in some studies to be a direct effector of the kinase domain of ABL. Thus, Rac activation in this model would not be expected to be dependent on CrkL activation. In that sense, our results are surprising but highly reproducible among leukemic animals. As CrkL activation has recently been reported to be dependent on a large multimeric protein complex that contains at least phosphoinositide-3 kinase (PI3K), docking protein 2 (DOK2), CrkL, Vav (a GEF responsible of Rac activation), and Rac (Nishihara et al., 2002; Sattler et al., 2001), we

suggest that the deficiency of Rac (Rac2 and especially the combined deficiency of Rac1 and Rac2) would also impair the activation of CrkL. The persistence of STAT5 signaling in $Rac1^{\Delta/\Delta};Rac2^{-/-}$ cells may also be important in at least two ways. First, we have previously shown that mice engrafted in a competitive repopulation assay lose contribution of $Rac1^{\Delta/\Delta};Rac2^{-/-}$ HSC/P shortly after Cre-mediated deletion of Rac1 sequences in the absence of Rac2 (Cancelas et al., 2005). Thus, persistent engraftment of p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta};Rac2^{-/-}$ cells over 100 days suggests that p210-BCR-ABL may be providing important signals affecting HSC retention and function in the BM in this setting. In this regard, STAT5 is a reasonable downstream candidate. STAT5 has been previously implicated in both the p210-BCR-ABL transformation phenotype and in normal HSC/P proliferation (Bradley et al., 2002; Sillaber et al., 2000; Ye et al., 2006; Ilaria and Van Etten, 1996). Independently, JAK proteins, particularly JAK2, which are known to activate STATs, have been implicated in p210-BCR-ABL transformation (Xie et al., 2001; Wilson-Rawls et al., 1996). Indeed, we have seen inhibition of proliferation in vitro of BM cells harvested from p210-BCR-ABL-expressing $Rac1^{\Delta/\Delta};Rac2^{-/-}$ mice that developed MPD using a JAK inhibitor (data not shown) (Thompson et al., 2002). Whether STAT5 activation in the absence of Rac1 and Rac2 is the result of direct signaling from p210-BCR-ABL via JAK (Xie et al., 2001) or the result of activation through Rac3 (or both) is yet to be determined.

In addition to the genetic data provided, our studies suggest that Rac proteins may prove to be useful molecular targets for pharmacologic intervention in human CML, particularly in p210-BCR-ABL-persistent and Imatinib-resistant disease. The combination of NSC23766 and Imatinib led to highly significant inhibition of proliferation of cells expressing the T315I mutant of p210-BCR-ABL. This first generation small molecule inhibitor of Rac is relatively nontoxic when administered chronically in vivo to mice (Cancelas et al., 2005) and appears to inhibit p210-BCR-ABL-induced leukemogenesis of primary murine and human cells in vivo.

Deregulated expression of Rac GTPases has previously been associated with several aspects of the leukemic phenotype. In particular, Rac1 has been shown to be an important downstream signaling component of p210-BCR-ABL (Skorski et al., 1998). p210-BCR-ABL-transduced Ba/F3 cells exhibit increased F-actin staining and an increased formation of filopodia and pseudopodia, reflecting elevated Rac1 activity (Salgia et al., 1997). In addition, a dominant-negative Rac1 mutant has been shown to inhibit p210-BCR-ABL-induced transformation in this cell line, and a signal cascade linking Abl kinase, phosphorylated Sos-1, and Rac-dependent phenotypes has been proposed (Sini et al., 2004). Harnois et al. reported that a stable complex could form between p210-BCR-ABL and multiple Rho GTPases and that Rac1, Rac2, Rho, and Cdc42 could be activated by p210-BCR-ABL, possibly through the Dbl homology domain of Bcr or activation of Vav as a guanine nucleotide exchange factor (GEF) that is associated with the complex (Harnois et al., 2003). However, these previous studies do not elucidate which specific Rac proteins are crucial in leukemic transformation and suffer from the lack of in vivo data on primary hematopoietic cells. The data presented here implicate Rac2, the combinatorial loss of both Rac1 and Rac2, and compensatory activation of Rac3 in leukemia development in p210-BCR-ABL disease. Our current data indicate that Rac GTPases are critical for p210-BCR-ABL-mediated transformation and, therefore, provide important targets for new therapy in CML.

EXPERIMENTAL PROCEDURES

Cell Lines

Stable Ba/F3 cell lines expressing full-length p210-BCR-ABL with the T315I point mutation and parental Ba/F3 cells were maintained as previously described (La Rosee et al., 2002).

Mice

The generation of C57Bl/6 Cre-transgenic ($Cre\text{-}^{Tg+}$);$Rac1^{flox/flox}$, $Cre^{Tg+};Rac1^{flox/flox};Rac2^{-/-}$, and $Cre^{Tg+};Rac2^{-/-}$ mice has been previously described (Gu et al., 2003; Cancelas et al., 2005). Animals used in these experiments were littermates. NOD/LtSz-scid/scid (NOD/SCID) mice, 5–6 weeks of age, were bred and housed under specific pathogen-free conditions in a laminar air flow unit and supplied with sterile food and drinking water containing doxycycline (6 mg doxycycline per gram of food, Bioserve Biotech, Laurel, MD) ad libitum. Housing, care, and all animal experimentation were done in conformity with protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Human Specimens

Normal BM and CML BM and PB were obtained through Institutional Review Board-approved protocols and donor informed consent from either Oregon Health Science University or Cincinnati Children's Hospital Medical Center. Material from therapeutic leukaphereses from two CML patients was submitted to CD34 selection by CliniMACS (Miltenyi Biotec Inc, Auburn, CA). Postselection purities were 99.6% and 99.7%. Interphase FISH showed that 98.6% and 99.4% of CD34+ cells carried the t(9,22) translocation, respectively.

Retroviral Vectors and Generation of Retroviral Stock

The retroviral vectors used have been described previously (Zhao et al., 2001; Gu et al., 2003; Williams et al., 2000). Generation of retroviral supernatants is described in Supplementary Experimental Procedures.

Bone Marrow Harvest, Transduction, and Transplantation

C57Bl/6 $Cre\text{-}^{Tg+}$;WT, $Cre\text{-}^{Tg+};Rac1^{flox/flox}$, $Cre^{Tg+}/Rac1^{flox/flox};Rac2^{-/-}$, and $Cre^{Tg+};Rac2^{-/-}$ mice were injected with 5-fluorouracil (5-FU, American Pharmaceutical Partners, Inc, Schaumburg, IL; 150 mg/kg i.p.) to enrich for HSC. BM cells were harvested as previously described (Gu et al., 2001) and stimulated with IMDM containing 2 mM L-glutamine, 10% FCS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 100 ng/ml AMP-4, 100 ng/ml recombinant human granulocyte-colony stimulating factor (G-CSF), and 100 ng/ml recombinant rat stem cell factor (rr-SCF, all Amgen, Thousand Oaks, CA) (cytokine-containing medium). Transduction of 5-FU-treated, LDBM cells was performed as previously described (Williams et al., 2000; Hanenberg et al., 1996). Two days posttransduction, $EGFP^+$ sorted cells were used either for transplantation or in vitro assays. For transplantation, a total of 50,000–75,000 EGFP-positive transduced BM cells were transplanted into lethally irradiated (1175 cGy, split-dose) 6–8 week old C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) in the presence of 500,000 freshly isolated C57Bl/6 erythrocyte-lysed BM cells.

Hematological and Pathological Examination of Transplanted Mice

Animals were bled weekly starting ten days posttransplant. Complete blood counts and leukocyte differentials were determined and disease progression was monitored. Animals in which the percentage of $EGFP^+$ cells in the peripheral blood fell below 1% for two consecutive time points were censored from the study, due to loss of engraftment. While no WT and $Rac1^{\Delta/\Delta};Rac2^{-/-}$ animals were removed from the study, four out of 12 $Rac1^{\Delta/\Delta}$ animals and 10 out 28 $Rac2^{-/-}$ animals were censored for this reason.

Homing and Engraftment Assays

See Supplemental Experimental Procedures online.

Generation of Rac1-Deficient Hematopoietic Cells

Cre-mediated recombination of floxed Rac1 sequences in hematopoietic cells was performed in vivo 10 days posttransplant as previously described (Cancelas et al., 2005; Mikkola et al., 2003). For in vitro deletion of the Rac1 floxed gene, LDBM cells were cotransduced with either MIEG3 and MSCV-Cre-YFP or MSCV-p210-BCR-ABL-EGFP and MSCV-Cre-YFP and were sorted for $EGFP^+/YFP^+$ expression.

In Vivo Administration of NSC23766

Ten days posttransplant, Alzet osmotic pumps (Model 2002, Durect, Cupertino, CA) containing either NSC23766 (2 pumps, 100 mM/pump) or PBS control at a flow rate of 0.5 μl/hr for 14 days were subcutaneously implanted into recipient mice. For NOD/SCID mice, mice were transplanted with a total of 10×10^6 $CD34^+$ cells per mouse and pumps were implanted on day +16 posttransplantation.

Proliferation Assays

Cell proliferation was assessed by either thymidine incorporation assays (transduced LDBM cells, $Rac1^{\Delta/\Delta};Rac2^{-/-}$ BM cells expressing p210-BCR-ABL, and Ba/F3-pSRC-T315I cells), cell counts (transduced LDBM cells and $Rac1^{\Delta/\Delta}/Rac2^{-/-}$ BM cells expressing p210-BCR-ABL), or MTS assays (transduced LDBM cells, Promega, Madison, WI), which are described in detail in Supplemental Experimental Procedures.

Rac Activation Assays

Splenocytes from transplanted mice or purified human CD34+ cells were processed for the preparation of protein extracts. The generation of protein extracts and Rac activation assays and electrophoresis are described in Supplemental Experimental Procedures.

Immunoblotting

Aliquots (25 μg) of protein extracts from the spleens of mice expressing either MIEG3 or MSCV-p210-BCR-ABL-EGFP were separated by electrophoresis on 7.5% or 12% SDS-PAGE gels under reducing conditions. Membranes were probed with antibodies specific for phospho-p42/p44 MAPK (1:1000), phospho-Akt (1:1000), phospho-p38 MAPK (1:1000), phospho-JNK (1:1000), phospho-CrkL (1:1000), phospho-STAT5 (1:1000), and β-actin (1:5000) (Cell Signaling) To examine phosphorylation of p210-BCR-ABL, BM cells from leukemic animals were lysed in high pH lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$ 10% glycerol, 20 mM Tris [pH 8.0]) supplemented with Complete protease inhibitor cocktail (Roshe Diagnostics, Indianapolis, IN) to which 1.25% of 10 M NaOH was added immediately before lysis. Cells were incubated on ice for 10 min, clarified at 12,000 × g for 3 min, and lysates were separated on a 7% SDS-PAGE gel. Membranes were probed with antiphosphotyrosine (4G10, Upstate, Charlottesville, VA) and anti β-actin antibodies. Blots were then incubated with a secondary antibody conjugated to HRP and directed against mouse or rabbit IgG (Cell Signaling Technology) and reactive proteins were visualized with LumiGLO (Upstate, Charlottesville, VA).

Flow Cytometric Analysis

Flow cytometric analysis is described in Supplemental Experimental Procedures.

Bone Marrow Hematopoietic Progenitor Assays

Colony-forming units-granulocyte/macrophage (CFU-GM) quantitation assays in methylcellulose have been previously described (Cancelas et al., 1998, 2005).

LAM-PCR

For clonality analysis of the p210-BCR-ABL insertion sites of leukemic animals, we performed LAM-PCR as previously described (Schmidt et al., 2003).

Statistical Analysis

Statistical analysis was performed using the unpaired Student's t test except for survival curves where the log P rank test was used. Values of $p < 0.05$ were considered significant.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, three supplemental tables, and three supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/5/467/DC1/.

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Supplemental Data

Rac Guanosine Triphosphatases Represent Integrating Molecular Therapeutic Targets for BCR-ABL-Induced Myeloproliferative Disease

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Supplemental Experimental Procedures

Generation of retroviral stock. Retroviral supernatant was generated from ecotrophic Phoenix cells (ATCC, Manassas, VA)(Williams, et al., 2000) that were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FCS and 1% P/S. Cells were transfected with either MIEG3, MSCV-p210-BCR-ABL, or MSCV-Cre-YFP using calcium phosphate transfection (Invitrogen, Carlsbad, CA) following the manufacturer's instructions with slight modifications. Briefly, 8 μg of plasmid DNA, 10 μg of Gag, and 3 μg of Envelope were mixed with 36 μL of $CaCl_2$ and added to sterile H_20 to a final volume of 300 μL. HEPES Buffered Saline (HBS, 2X) at a volume of 300 μL was slowly added to the DNA solution, and the mixture was bubbled thoroughly with a pipette for 1 minute. After a 30 minute incubation at room temperature, the solution was mixed with media containing chloroquine and added to the Phoenix cells. Cells were incubated overnight at 37˚C with 5% CO_2, after which fresh media was added to the plates. Retroviral supernatant

was harvested 36, 48, 60, and 72 hours after transfection and stored at -80˚C until use.

Homing and Engraftment assays. 5-FU (4 day) LDBM cells from WT or Rac1$^{flox/flox}$;Rac2$^{-/-}$ animals that were transduced with either MIEG3 vector control or MSCV-p210-BCR-ABL were labeled with the lipophilic dye PKH26 according to manufacturer's instructions. PKH26+/EGFP+ cells were sorted in a FACSVantage SE DiVa (BD, San Jose, CA) and 80,000-250,000 cells were intravenously injected into 11.75 Gy-irradiated C57Bl/6 mice (split dose; 7 + 4.75 Gy, 3 hours apart at 58-63 cGy/min). Sixteen hours after injection, mice were sacrificed and femorae, tibiae, iliac crest and spleens were retrieved aseptically. Single cell suspensions of BM were prepared as described above. The BM homing efficiency was calculated on the basis of the number of input and output PKH26+/EGFP+ cells in BM assuming that both femurs, tibiae and iliac crest contained 25% of all the murine BM(Boggs, 1984). For engraftment assays, 50,000 EGFP+ BCR-ABL-expressing WT or Rac1$^{flox/flox}$;Rac2$^{-/-}$ LDBM cells and 500,000 unmanipulated whole bone marrow cells (after RBC lysis) were transplanted into lethally irradiated 6-8 week old C57Bl/6 mice. Eighteen days post-transplant, animals were sacrificed and bone marrow cells were stained with a cocktail of antibodies for lineage, Sca-1, and c-Kit molecules as described previously(Gu, et al., 2003, Cancelas, et al., 2005).

Proliferation assays. Cell proliferation was assessed by either thymidine incorporation assays (transduced LDBM cells, $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ bone marrow cells expressing p210-BCR-ABL, Ba/F3-pSRC cells, and Ba/F3-pSRC-T315I cells), cell counts (transduced LDBM cells and $Rac1^{\Delta/\Delta}$/$Rac2^{-/-}$ bone marrow cells expressing p210-BCR-ABL), or MTS assays (transduced LDBM cells, Promega, Madison, WI). After transduction and sorting, EGFP and EGFP/YFP-positive LDBM cells were plated in IMDM supplemented with 10% FCS, 2 mM L-glutamine, 1% P/S, 100 ng/ml G-CSF, 100 ng/ml AMP-4, and 100 ng/ml SCF and incubated overnight at 37˚C in 5% CO2 in preparation for the proliferation assays. For thymidine incorporation assays and cell counts, cells were starved in IMDM supplemented with 1% FCS for six hours, then were counted and seeded in six replicates at a density of 5,000 (cell counts) or 25,000 (thymidine incorporation assays) cells per well with or without 100ng/ml rr-SCF in IMDM with 10% FCS and 1% P/S. In some experiments, cells were incubated in the presence of increasing concentrations of NSC23766, a Rac-specific inhibitor(Gao, et al., 2004). After twenty-four or forty-eight hours, cells were counted or were pulsed for six hours with 1 μCi [^{3}H]-thymidine per well. Cells were harvested with an automated cell harvester (Packard Bioscience, Perkin Elmer, Boston, MA) and thymidine incorporation was determined using a liquid scintillation counter (Beckman LS-6500, Beckman Coulter, Fullerton, CA). For MTS assays, 10,000 MIEG3 or BCR-ABL-transduced cells were incubated in 96-well culture plates for 48 hours in the presence of increasing concentrations of

NSC23766, then reacted with 20 µl MTS/PMS solution at 37 °C for 3 hours. The absorbance of the reaction product, soluble formazan, was read at 490 nm.

Rac activation assays. Splenocytes from transplanted mice or purified human CD34+ cells were processed for the preparation of protein extracts. Cells were pelleted and flash-frozen, and the proteins were isolated with 1X Magnesium Lysis/Wash Buffer (Upstate Biotechnology, Lake Placid, NY) supplemented with 25 mM NaF and 1 mM Na_3VO_4. GTP-bound Rac from the lysates was immunoprecipitated with agarose beads coated with the Rac binding domain of Pak1 (Upstate Biotechnology), according to the manufacturer's instructions. The immunoprecipitates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 12% gel (Bio-Rad, Hercules, CA) and were transferred to nitrocellulose. Rac isoforms were detected by using anti-Rac1 antibody (Upstate Biotechnology), anti-Rac2 antibody (Novus, Littleton, CO) or anti-Rac3 antibody (A. D. Cox and P. J. Keller, University of North Carolina). Total active Rac was detected by immunoblotting with a mouse monoclonal antibody against Rac GTPases (BD Transduction Laboratories)**.** All incubations were carried overnight at 4˚C, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (HRP) and directed against mouse (Cell Signaling Technology, Beverly, MA), rabbit (Cell Signaling) or goat IgG (Novus) for 1 hour at room temperature. Reactive proteins were visualized with a chemiluminescent detector system (LumiGLO Reagent and Peroxide, Cell Signaling Technology, Beverly, MA).

Flow cytometric analysis. The following fluorochrome-labeled monoclonal antibodies were purchased from BD Pharmingen (San Jose, CA): Gr-1(Ly6G), Mac-1 (CD11b, cloneM1/70), CD45R (B220, clone RA3-6B2), CD3e (clone 145-2C11) for leukemia phenotyping. Lineage staining was performed as previously described(Gu, et al., 2003). CD44 expression was analyzed by staining with anti-H-CAM antibody (clone IM7). Peripheral blood cells, splenocytes, and bone marrow cells were labeled for fifteen minutes in phosphate-buffered saline (PBS) in the dark, washed, and resuspended in isoton solution. Flow cytometry data were acquired on a FACSCalibur bench-top flow cytometer (Becton Dickinson). Live cells were gated for analysis by forward and side scatter signals.

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Table S1:

Recipient Mice	Survival at Day 100	Spleen weight	Pulmonary Hemorrhage
WT MIEG3	24/24	0.089 ± 0.03	0/24
$Rac1^{\Delta/\Delta}$ MIEG3	8/8	0.176 ± 0.17	0/8
$Rac2^{-/-}$ MIEG3	20/21	0.102 ± 0.04	0/21
$Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ MIEG3	13/14	0.131 ± 0.08	0/14
WT BCR-ABL	0/30	0.410 ± 0.12	17/18
$Rac1^{\Delta/\Delta}$ BCR-ABL	0/8	0.333 ± 0.06	8/8
$Rac2^{-/-}$ BCR-ABL	1/18	0.553 ± 0.19	12/13
$Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ BCR-ABL	8/19	0.312 ± 0.17	4/16

Table S2:

Mouse genotype	Days Post-transplant Leukemia First Observed	Phenotype/ % Observed
WT BCR-ABL	16	Myeloid (Gr-1/Mac-1) 100%
$Rac1^{\Delta/\Delta}$ BCR-ABL	17	Myeloid (Gr-1/Mac-1) 100%
$Rac2^{-/-}$ BCR-ABL	21	Myeloid (Gr-1/Mac-1) 100%
$Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ BCR-ABL—early disease	25	Myeloid (Gr-1/Mac-1) 67% Lymphoid (B220) 33%
$Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ BCR-ABL—late disease	70	Myeloid (Gr-1/Mac1) 62% Lymphoid (B220) 23% Myeloid/Lymphoid 15%

Table S3:

Pump	Plasma Concentration (μM)	WBC ($x10^3/mm^3$)
PBS	0.055	4.26
PBS	0.006	6.34
PBS	0.003	4.04
Average ± SD – PBS	**0.02 ± 0.03**	**4.88 ± 1.27**
NSC23766	3.1	6.32
NSC23766	0.95	10.5
NSC23766	1.09	4.88
Average ± SD - NSC23766	**1.71 ± 1.20**	**7.23 ± 2.92**

Figure S1. Homing, engraftment and CD44 expression appear not to be impaired by Rac deficiency in p210-BCR-ABL-expressing HSC/P. **A.** Expression of the $Rac1^{flox}$ alleles and deletion of Rac2 do not appear to inhibit homing and engraftment of BCR-ABL-expressing cells. p210-BCR-ABL-expressing ($EGFP^{+}$) WT and $Rac1^{flox/flox}$;$Rac2^{-/-}$ cells were labeled with PKH26. Sorted PKH26+/$EGFP^{+}$ cells were transplanted into lethally irradiated C57Bl/6 mice. Sixteen hours later, mice were sacrificed and the number of PKH26-labeled cells in the bone marrow was determined. Data represent mean ± SD, n = 3-7 mice per group, p=N.S. **B.** Short-term engraftment of $EGFP^{+}Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ bone marrow HSC/P harvested from mice transplanted with p210-BCR-ABL-expressing WT or $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ BM cells 18 days post-transplant. Data represent mean ± SD, n = 4 mice per group. **C & D.** CD44 expression was analyzed in p210-BCR-ABL-expressing cells *in vitro* and *in vivo*. **C.** Transduced and sorted p210-BCR-ABL (p210-BCR-ABL-EGFP) and (Cre-YFP) WT and $Rac1^{\Delta/\Delta}$;$Rac2^{-/-}$ BM cells were analyzed for CD44 expression. **D.** p210-BCR-ABL-transduced WT and $Rac1^{flox/flox}$, $Rac2^{-/-}$ BM cells were labeled with PKH26. PKH26+/EGFP+ BM cells were transplanted into lethally irradiated recipients and retrieved from femorae and tibiae after 16 hours post-transplantation. CD44 expression was analyzed on PKH26+/EGFP+ homed BM cells (p=N.S.). Data represent mean ± SD, n=3 mice per group.

Thomas et al., Suppl. Fig. 1

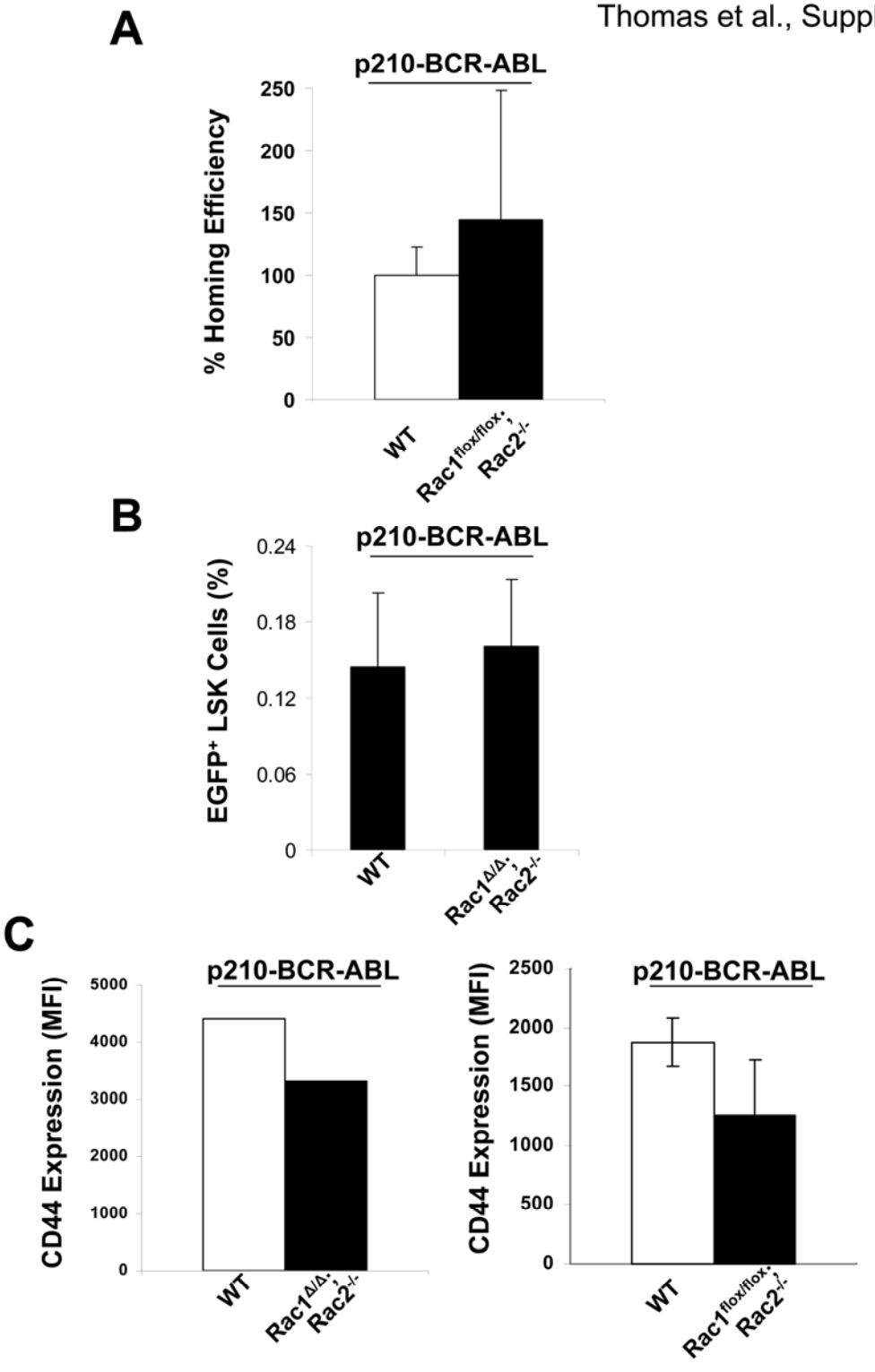


Figure S2. NSC23766 inhibits Rac1, Rac2 and Rac3 activation of p210-BCR-ABL-expressing wild-type BM cells. 5-FU-treated LDBM cells were transduced with p210-BCR-ABL, serum-starved overnight (16-18 hours) and treated with NSC23766 for 30 minutes. Cells were lysed and lysates submitted to PBD pull-down assays (Rac1-GTP, Rac2-GTP and Rac3-GTP) or loading control immunoblot (β-actin).

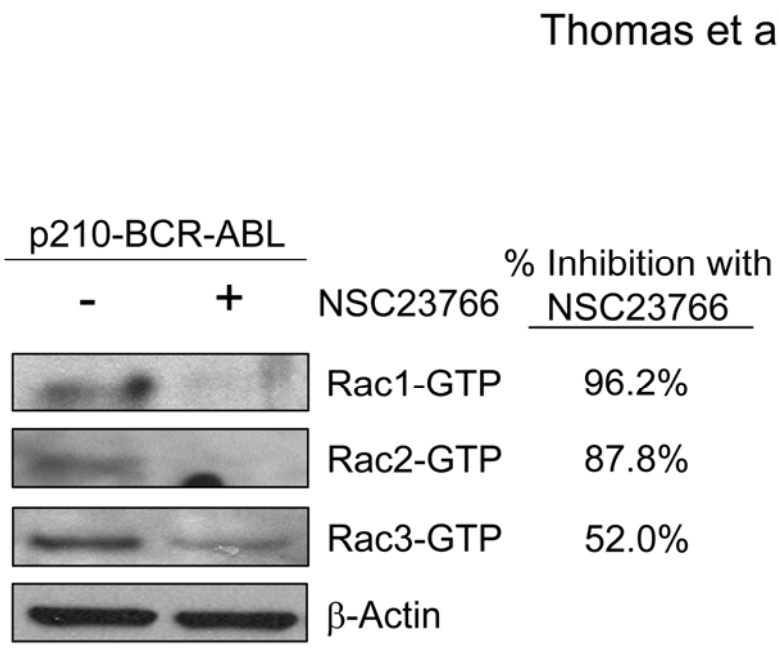
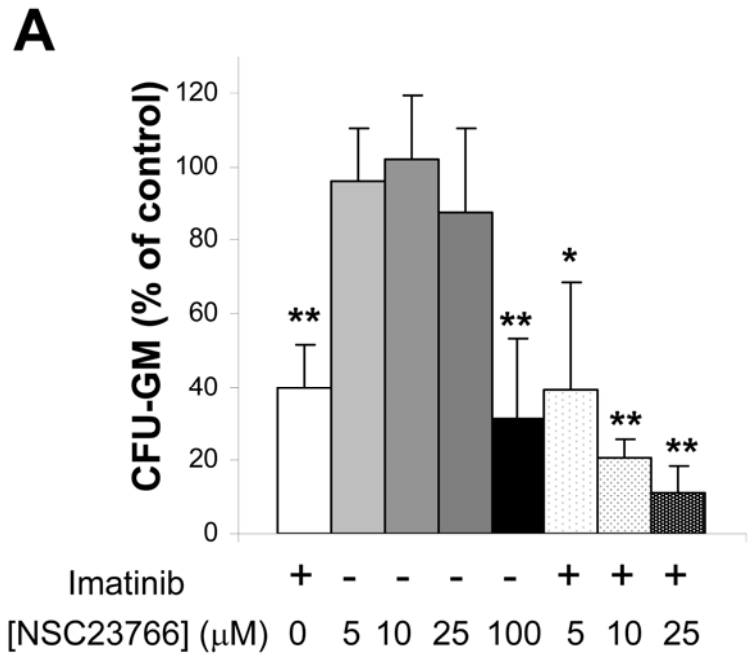


Figure S3. NSC23766 at 10-25 μM concentrations does not appear to significantly alter the proliferation of normal human bone marrow specimens, while 100 μM NSC23766 impairs human progenitor proliferation similar to 1 μM imatinib. Analysis of CFU-GM **(A)** or BFU-E **(B)** formation of normal human BM cells in the presence of imatinib (1 μM) and/or NSC23766 at different concentrations (n=5). Data represent mean ± SD of the level of inhibition reached for each specific specimen. * $p < 0.01$, ** $p < 0.001$.


A
CFU-GM (% of control)
120
100
80
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40
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0
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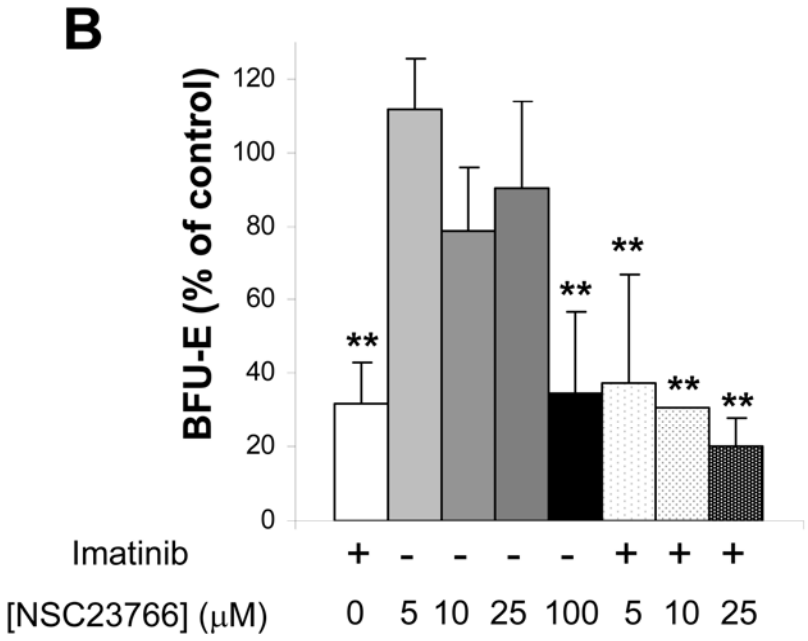
Imatinib
+ - - - - + + +
[NSC23766] (μM) 0 5 10 25 100 5 10 25


B
BFU-E (% of control)
120
100
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CHAPTER TWENTY-SEVEN

Rho GTPases and Regulation of Hematopoietic Stem Cell Localization

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Abstract

Bone marrow engraftment in the context of hematopoietic stem cell and progenitor (HSC/P) transplantation is based on the ability of intravenously administered cells to lodge in the medullary cavity and be retained in the appropriate marrow space, a process referred to as homing. It is likely that homing is a multistep process, encompassing a sequence of highly regulated events that mimic the migration of leukocytes to inflammatory sites. In leukocyte biology, this process includes an initial phase of tethering and rolling of cells to the endothelium via E- and P-selectins, firm adhesion to the vessel wall via integrins that appear to be activated in an "inside-out" fashion, transendothelial

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migration, and chemotaxis through the extracellular matrix (ECM) to the inflammatory nidus. For HSC/P, the cells appear to migrate to the endosteal space of the bone marrow. A second phase of engraftment involves the subsequent interaction of specific HSC/P surface receptors, such as $\alpha_4\beta_1$ integrin receptors with vascular cell–cell adhesion molecule-1 and fibronectin in the ECM, and interactions with growth factors that are soluble, membrane, or matrix bound. We have utilized knockout and conditional knockout mouse lines generated by gene targeting to study the role of Rac1 and Rac2 in blood cell development and function. We have determined that Rac is activated via stimulation of CXCR4 by SDF-1, by adhesion via β_1 integrins, and via stimulation of c-kit by the stem cell factor—all of which involved in stem cell engraftment. Thus Rac proteins are key molecular switches of HSC/P engraftment and marrow retention. We have defined Rac proteins as key regulators of HSC/P cell function and delineated key unique and overlapping functions of these two highly related GTPases in a variety of primary hematopoietic cell lineages *in vitro* and *in vivo*. Further, we have begun to define the mechanisms by which each GTPase leads to specific functions in these cells. These studies have led to important new understanding of stem cell bone marrow retention and trafficking in the peripheral circulation and to the development of a novel small molecule inhibitor that can modulate stem cell functions, including adhesion, mobilization, and proliferation. This chapter describes the *biochemical footprint* of stem cell engraftment and marrow retention related to Rho GTPases. In addition, it reviews abnormalities of Rho GTPases implicated in human immunohematopoietic diseases and in leukemia/lymphoma.

1. Basic Mechanisms of Hematopoietic Stem Cell and Progenitor (HSC/P) Homing and Retention in Bone Marrow (BM)

Bone marrow engraftment in the context of HSC/P transplantation is based on the ability of intravenously administered cells to lodge in the medullary cavity and be retained in the appropriate marrow space, a process referred to as homing. It is likely that homing is a multistep process, encompassing a sequence of highly regulated events that mimic the migration of leukocytes to inflammatory sites. In leukocyte biology, this process includes an initial phase of tethering and rolling of cells to the endothelium via E- and P-selectins, firm adhesion to the vessel wall via integrins that appear to be activated in an ''inside-out'' fashion, transendothelial migration, and chemotaxis through the extracellular matrix (ECM) to the inflammatory nidus (Butcher and Picker, 1996; Peled *et al.*, 1999a; Springer, 1994). For HSC/P, the cells appear to migrate to the endosteal space of the bone marrow (Driessen *et al.*, 2003; Gong, 1978; Nilsson *et al.*, 2001; Wilson and Trumpp, 2006). A second phase of engraftment involves the

subsequent interaction of specific HSC/P surface receptors, such as $\alpha_4\beta_1$ integrin receptors with vascular cell–cell adhesion molecule-1 (VCAM-1) and fibronectin in the ECM, and interactions with growth factors that are soluble, membrane, or matrix bound (Williams *et al.*, 1991a,b). HSC/P can be temporarily detected in other organs such as liver, lung, and kidneys after intravenous infusion but disappear from these sites within 48 h after transplantation. In contrast, the retention of HSC/P in BM is sustained and appears specific (Papayannopoulou *et al.*, 2001a).

Some of the factors that influence this specific retention of HSC/P in the bone marrow have been defined recently and appear to involve the interplay among chemokines, growth factors, proteolytic enzymes, and adhesion molecules (Papayannopoulou, 2003).

Among the chemokines, stromal derived factor-1α (SDF-1α) and its receptor, the G-protein-coupled seven-span transmembrane receptor, CXCR4, play key roles in HSC trafficking and repopulation (Lapidot and Kollet, 2002). SDF-1α is expressed by both human and murine BM endothelium and stroma (Nagasawa *et al.*, 1998; Peled *et al.*, 1999a) and acts as a powerful chemoattractant of HSC/P (Aiuti *et al.*, 1997; Wright *et al.*, 2002). SDF-1α may also regulate the survival of HSC/P (Broxmeyer *et al.*, 2003; Lataillade *et al.*, 2000). SDF-1α induces the integrin-mediated firm arrest of hematopoietic progenitor cells and facilitates their transendothelial migration (Peled *et al.*, 1999a, 2000) and regulates HSC/P homing (Kollet *et al.*, 2001) and BM engraftment (Peled *et al.*, 1999b). Furthermore, SDF-1α is also required for the retention of murine HSC/P within the BM (Ma *et al.*, 1999; Nagasawa *et al.*, 1996). We have demonstrated that Rac proteins are activated by SDF-1 in HSC/P and that Rac-deficient HSC/P do not respond to SDF-1 (Fig. 27.1).

Among growth factors, a critical component for HSC/P survival and engraftment is the stem cell factor (SCF), which is expressed on BM stromal cells and is the ligand for the receptor tyrosine kinase, c-kit. A transmembrane isoform of SCF, membrane-bound SCF (membrane, mSCF), has been shown to be critical in the lodgment and retention of HSC within the hematopoietic microenvironment, although it does not appear to play a role in the homing of transplanted cells to BM (Driessen *et al.*, 2003). In addition, it appears that c-kit activation is differentially affected by soluble versus membrane-bound SCF and that mSCF appears to enhance maintenance of long-term hematopoiesis *in vitro* (Miyazawa *et al.*, 1995; Toksoz *et al.*, 1992) and induces overexpression of CXCR4 (Kollet *et al.*, 2001; Peled *et al.*, 1999b). Our studies of SCF-stimulated cell proliferation demonstrate that Rac activation is a critical component of c-kit signaling (see Fig. 27.1).

A third factor important for homing and engraftment of HSC/P are the integrin-mediated adhesion molecules. Among them, β_1 integrins are probably the best characterized, and inhibition of integrin function leads to defective medullary engraftment (Papayannopoulou and Craddock, 1997;

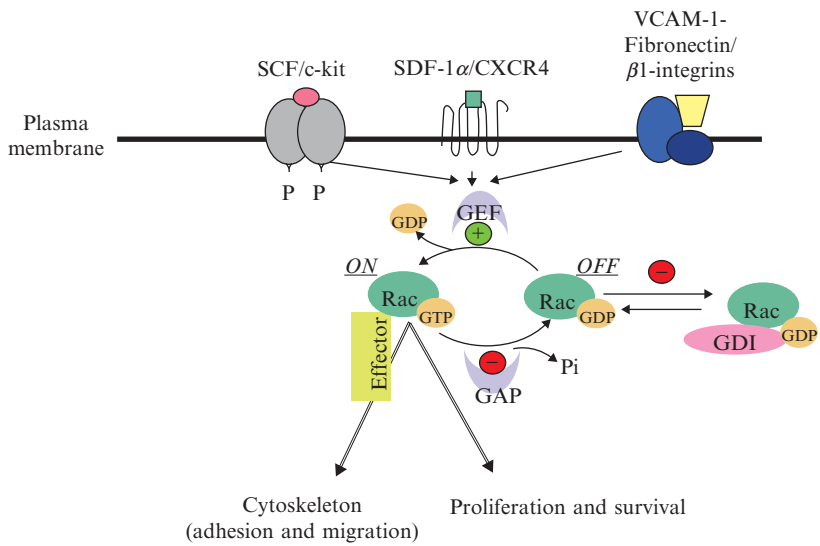

Figure 27.1 Rac GTPases integrate signals from multiple surface receptors involved in HSC engraftment and retention.

Papayannopoulou *et al.*, 1995, 2001a; Scott *et al.*, 2003; van der Loo *et al.*, 1998; Williams *et al.*, 1991a). As detailed later, Rac-deficient HSC/P show significantly defective adhesion to fibronectin (Cancelas *et al.*, 2005; Gu *et al.*, 2003; Yang *et al.*, 2001) (see Fig. 27.1).

2. Basic Mechanisms of HSC/P Mobilization and Trafficking

Hematopoietic stem cell and progenitor mobilization is also a dynamic and complex process (Kronenwett *et al.*, 2000; Rafii *et al.*, 2002; Thomas *et al.*, 2002). HSC/P must exit the stem cell niche in the BM (presumably; see comments later), migrate through the marrow sinusoidal endothelium, and gain access to the blood. Circulating HSC/P and BM-adherent HSC/P appear to be interchangeable. Studies utilizing parabiotic mice have demonstrated that HSC/P can leave their niche without induction, traffic through the bloodstream, and finally migrate into BM of the conjoined animal (Abkowitz *et al.*, 2003; Wang *et al.*, 2003; Warren *et al.*, 1960; Wright *et al.*, 2001). This suggests that HSC/P trafficking is a physiological process. If so, circulating HSC/P would be predicted to move into the BM microenvironment through transendothelial migration directed by chemoattractants and ultimately anchor within the extravascular BM space where

proliferation and differentiation occur. In this process, adhesion molecules, chemokine receptors, and integrin signaling require signal integration that drives cytoskeleton rearrangements and regulates gene expression, cell survival, and cell cycle activation. An additional proposed HSC location in the marrow is the vascular niche, where HSC would be attached to the fenestrated endothelium of the BM specialized vessels, so-called sinusoids (Kiel *et al.*, 2005). Such an outlook is supported by evidence that Rac-deficient HSC with profoundly defective cell migration due to loss of the combined function of CXCR4, β_1 integrins, and c-kit signaling pathways can be mobilized in large numbers (Cancelas *et al.*, 2005) [for a commentary, see Cancelas *et al.* (2006)]. The BM sinusoids express molecules important for HSC mobilization, homing, and engraftment, including chemokines such as CXCL12 (ligand for CXCR4) and adhesion molecules such as endothelial-cell (E)-selectin and vascular cell-adhesion molecule 1 (VCAM-1). These findings have given additional microanatomical clarity to the concept of stem cell niches as spatial structures in which HSC reside, self-renew, and differentiate.

At the molecular level, the interaction between SDF-1α and the G-coupled chemokine receptor CXCR4 has been recognized as pivotal in stem cell mobilization. As HSC/P are known to migrate toward a SDF-1α (Sweeney *et al.*, 2002), it has been suggested that treatment with granulocyte colony-stimulating factor-1 (G-CSF), cyclophosphamide, or interleukin (IL)-8 leads to a reduction of SDF-1β in BM, resulting in a positive gradient in blood and induction of HSC/P migration toward PB. Raising the plasma levels of SDF-1α by intravenous injection of SDF-1α-expressing adenovirus (Hattori *et al.*, 2001) or sulfated polysaccharides (Sweeney *et al.*, 2002) or by inhibition of the CXCR4 receptor (Devine *et al.*, 2004; Liles *et al.*, 2003; Tavor *et al.*, 2004) leads to mobilization of HSC/P. G-protein inhibition by pertussis toxin (Papayannopoulou *et al.*, 2003) induces a similar mobilization effect, probably by interfering with the CXCR4 signaling pathway. It has been suggested that bone expression of CXCL12 (another ligand for CXCR4) is regulated by G-CSF-induced β_2-adrenergic signals that modify osteoblast protein expression and shape (Katayama *et al.*, 2006).

Functional blocking of $\alpha_4\beta_1$ integrin (receptor for VCAM-1 and fibronectin) alone or together with $\alpha_l\beta_2$ integrins or the functional blocking of the β_2 integrin leukocyte function-associated antigen-1 by antibodies results in the mobilization of HSC/P (Craddock *et al.*, 1997; Papayannopoulou *et al.*, 2001b). HSC/P accumulate in the PB soon after gene deletion in inducible $\alpha_4\beta_1$ integrin-deficient mice. Although their numbers gradually stabilize at a lower level, progenitor cell influx into the circulation continues at above-normal levels for more than 50 weeks with a concomitant progressive accumulation of spleen HSC/P (Scott *et al.*, 2003).

Playing an important and independent role in HSC/P mobilization is the interaction between SCF and its receptor, c-kit. SCF/c-kit interaction

plays a critical role in G-CSF-mediated mobilization (Heissig *et al.*, 2002; Levesque *et al.*, 2003), and SCF in combination with G-CSF has been shown to enhance HSC/P mobilization (McNiece and Briddell, 1995). As mentioned earlier, tm-SCF has been shown to be critical in retaining HSC/P in BM (Driessen *et al.*, 2003).

3. Rho GTPases

Almost all Rho family GTPases influence actin polymerization within the cell via specific or shared effectors and are thereby implicated in reorganization of the cytoskeleton, migration, and adhesion. However, Rho proteins regulate a multitude of other cellular functions. Among these are apoptosis and survival, cell cycle progression, and genomic stability. The activity of individual Rho proteins can be regulated by multiple guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs), which are cell and agonist specific. Indeed, Rho GTPases such as Rac appear to integrate signaling from multiple receptors in individual cells. For instance, as mentioned earlier and detailed later, we have shown in hematopoietic cells that Rac is activated by stimulation of CXCR4 via SDF-1, adhesion via β_1 integrins, and stimulation of c-kit via SCF—all pathways involved in stem cell engraftment (Cancelas *et al.*, 2005; Gu *et al.*, 2003; Yang *et al.*, 2001). In addition, Rho family members recognize both unique and shared effectors. This, at least in part, explains the diversity of cellular functions influenced by a single Rho GTPase but also presents significant complexities in developing an understanding of the physiological roles of these proteins, particularly if studies utilize cell lines and expression of dominant negative (DN) or constitutive active (CA) mutants, which generally lack specificity among related GTPases. We have exploited mouse knockouts to study the function of Rac GTPases in hematopoiesis in an attempt to circumvent the problems associated with these DN and CA mutants.

Using genetic approaches (primarily gene targeting in mice), we have specifically implicated PAK, POR1, and STAT5 in Rac effector functions in primary hematopoietic cells and, depending on the specific lineage and agonist, found that Rac can activate p42/p44 and p38 ERKs, JNK, and Akt kinases (Cancelas *et al.*, 2005; Carstanjen *et al.*, 2005; Gu *et al.*, 2003; Roberts *et al.*, 1999; Yang *et al.*, 2000, 2001) (and preliminary data). In a similar manner to the Wiskott–Aldrich syndrome protein (WASp), a key downstream target of Rac, WAVE1/2 and insulin receptor substrate (IRS) p53, has been implicated by others (primarily Takenawa and Miki, 2001) in actin polymerization and assembly. IRSp53 is a linker between Rac and WAVE1/2 that adds specificity for the actin-related protein (Arp)2/3

complex activator in actin polymerization. WAVE1 is required for Rac-mediated dorsal membrane ruffling, whereas WAVE2 may be involved in Rac-induced peripheral ruffling during cell migration (Miki *et al.*, 2000; Suetsugu *et al.*, 2003). The induction of actin polymerization by WAVE is dependent on Arp2/3 via the VCA (verprolin homology, cofilin homology, acidic) domain of WAVE1/2 in a manner analogous to WASp. The VCA domain is a G-actin and Arp2/3-binding domain required for Rac/Cdc42-induced *de novo* actin nucleation and actin polymerization (reviewed in Takenawa and Miki, 2001). While the physiological relevance of these molecular links to Rac remains unknown, *de novo* actin nucleation appears critical for actin assembly at the leading edge of migrating cells and we hypothesize that this is of particular relevance to the migration of hematopoietic cells. In addition (and likely also relevant to the BM microenvironment), WAVE is essential for cell migration mediated through ECM in mouse embryo fibroblasts (Eden *et al.*, 2002).

Thus, the combination of unique and shared upstream activators and downstream effectors, which may be cell type specific, represents an important mechanism by which the same Rho GTPase regulates a variety of the aforementioned cellular processes. The utilization of a genetic approach and the study of primary cells in murine models have contributed greatly to a new understanding of both unique and overlapping functions of Rho GTPases in hematopoiesis.

A significant proportion of known Rho GTPases is expressed ubiquitously, while two Rho proteins show tissue-specific expression. This is particularly important in hematopoietic cells and has been studied extensively for the Rac subfamily. All members of this subfamily (Rac1, Rac2, and Rac3) show high sequence similarity. Rac1 is expressed ubiquitously, whereas Rac3 is expressed in nearly all cell lines examined thus far and is expressed at high levels in murine heart, placenta, pancreas, and brain. Rac2 is expressed in a hematopoietic-restricted fashion. Thus, hematopoietic cells are unique in that all three Rac proteins are coexpressed and also express RhoH, the only other identified hematopoietic-specific Rho GTPase. RhoH has been shown by us and others to modulate Rac signaling (Gu *et al.*, 2005b, 2006; Li *et al.*, 2002a). Despite this expression pattern and their significant homology, individual Rac proteins are responsible for unique functions in hematopoietic cells, as gene-targeted mice deficient in each protein have measurable and distinct phenotypes. This has been well documented by our group and others using gene-targeted mice to examine the role of Rac1 versus Rac2 in hematopoietic cells (Fig. 27.2) (and see later). Genetic deletion of Rac2 leads to a number of phenotypic changes in multiple hematopoietic lineages, including granulocytes (Abdel-Latif *et al.*, 2004; Carstanjen *et al.*, 2005; Filippi *et al.*, 2004; Glogauer *et al.*, 2003; Kim and Dinauer, 2001; Lacy *et al.*, 2003; Li *et al.*, 2002a; Roberts *et al.*, 1999), B cells (Croker *et al.*, 2002b; Walmsley *et al.*, 2003), T cells (Croker *et al.*, 2002a),

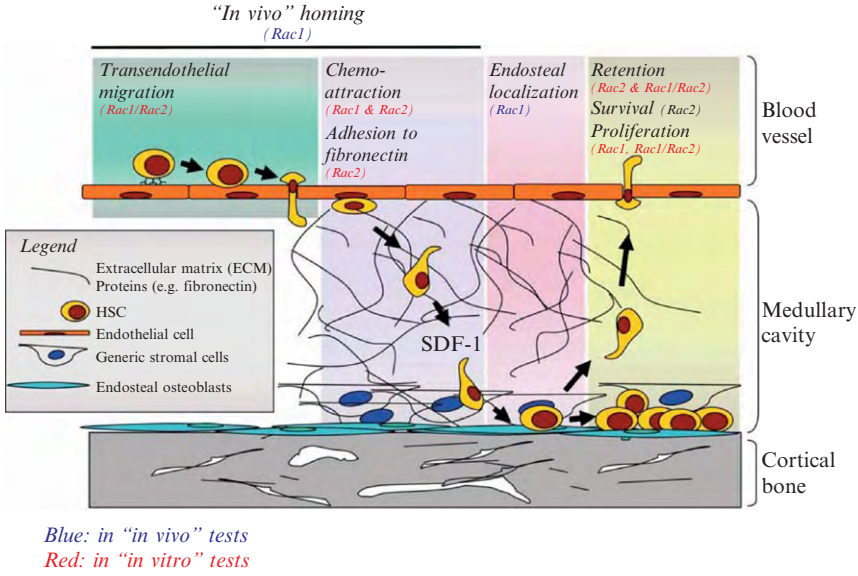

Figure 27.2 **Unique and overlapping roles of Rac1 versus Rac2 in hematopoiesis. Putative utilization of Rac1 and Rac2 in processes involved in homing, migration, endosteal localization, retention, and proliferation/survival is shown.**

mast cells (Gu *et al.*, 2002; Tan *et al.*, 2003; Yang *et al.*, 2003), eosinophils (Fulkerson *et al.*, 2005), and platelets (Akbar *et al.*, 2006), despite continued expression (and even a compensatory increase in expression in some cases) of Rac1. Considerable evidence shows that Rac2 and Rac1 regulate both separable and overlapping functions in nearly all lineages on the hematopoietic cells examined (Filippi *et al.*, 2004; Gu *et al.*, 2003; Walmsley *et al.*, 2003). Thus, Rac1 cannot compensate for the loss of Rac2 function in hematopoietic cells and vice versa.

In addition, Rho GTPases from different subgroups appear to demonstrate cross talk to regulate cellular responses. In previous studies, primarily in fibroblasts, introduction of constitutive active or dominant negative mutants of Cdc42, Rac, and RhoA was shown to affect activation or inhibition of each other. Growth factor receptor-induced activation of Cdc42 has been shown to activate Rac, which in turn stimulates Rho activity, resulting in cytoskeletal remodeling. RhoE has been shown to downregulate the activity of RhoA by activating p190RhoGAP. Similarly, as mentioned earlier, RhoH has been shown to repress Rac activity in lymphoid cell lines (Li *et al.*, 2002a) and cytokine-stimulated hematopoietic progenitor cells, resulting in reduced proliferation, increased apoptosis, and defective actin polymerization (Gu *et al.*, 2005a). In addition, we have demonstrated that expression of a patient-derived dominant negative Rac

GTPase, D57N, in hematopoietic cells not only inhibits Rac1 and Rac2 activities, but may also inhibit Cdc42 (Y. Gu and D. Williams, unpublished results). Additional genetic studies have demonstrated cross-talk between Rac and both Cdc42 (Yang *et al.*, 2001) and RhoA (Filippi *et al.*, manuscript in preparation).

4. Role of Rac1 and Rac2 GTPases in Hematopoiesis

Using gene-targeted mice, it has become evident that the Rho family of GTPases plays an important role in hematopoietic stem cell function. Rac activity has been demonstrated to be important for such diverse functions as retention in the bone marrow (Cancelas *et al.*, 2005; Yang *et al.*, 2001), long-term engraftment of HSC (Jansen *et al.*, 2005), and HSC mobilization (Gu *et al.*, 2003). Furthermore, in more committed hematopoietic cells, Rac activity is associated with B-lymphocyte development and signaling (Croker *et al.*, 2002b; Walmsley *et al.*, 2003), granulocyte chemotaxis and superoxide production (Abdel-Latif *et al.*, 2004; Carstanjen *et al.*, 2005; Filippi *et al.*, 2004; Glogauer *et al.*, 2003; Kim and Dinauer, 2001; Lacy *et al.*, 2003; Li *et al.*, 2002a; Roberts *et al.*, 1999), migration and degranulation of mast cells (Gu *et al.*, 2002; Yang *et al.*, 2000), differentiation of mature osteoblasts (Lax *et al.*, 2004), and maturation of TRAP-positive, pro-osteoclasts into multinucleated osteoclasts (Korhonen *et al.*, manuscript in preparation).

Rac1-deficient HSC/P stimulated with SCF demonstrate defective proliferative signaling from the c-kit receptor tyrosine kinase *in vitro* (Gu *et al.*, 2003). In contrast, loss of Rac2 activity leads to a pro-apoptotic phenotype in both mast cells and HSC/P in the presence of SCF (Gu *et al.*, 2002, 2003; Yang *et al.*, 2000). Rac integrates signals from β_1 and β_2 integrins and c-kit in HSC/P and mast cells (Gu *et al.*, 2002, 2003; Tan *et al.*, 2003). Signaling of c-kit to Rac is mediated through the GEF Vav (see later and preliminary data), although the specific Vav responsible for signaling to Rac in nonlymphoid hematopoietic cells remains largely unknown. Thus, overall, studies utilizing mouse mutants implicate Rac proteins downstream of CXCR4, c-kit, and β_1 and β_2 integrins, and Rac-deficient hematopoietic cells show loss of adhesion, migration, degranulation, changes in cell shape consistent with deregulated actin assembly, and defects in cell proliferation and survival linked to alterations in kinase pathways that are both lineage and agonist specific (for a complete review, see Cancelas *et al.*, 2006). Rac GTPases are thus important molecular switches controlling stem cell localization and retention in the marrow microenvironment, engraftment, and reconstitution in transplanted mice. These

proteins represent a novel molecular target to modulate hematopoietic cell functions (Nasser *et al.*, 2006), and we have developed a first-generation, small molecule inhibitor, NSC23766, which induces mobilization of HSC/P (Cancelas *et al.*, 2006).

5. Rac3 GTPase, a Newly Defined Member of the Rac Family Cloned from a Bcr-abl Transformed Cell Line

Rac3 is a third member of the Rac subfamily, which was originally identified from a chronic myelogenous leukemia cell line, and has been implicated in human breast cancer (Baugher *et al.*, 2005; Mira *et al.*, 2000), ovarian cancer (Morris *et al.*, 2000), cellular transformation (Keller *et al.*, 2005), and tumor invasion (Chan *et al.*, 2005). Rac3 has been shown to interact with the integrin-binding protein CIB and promotes integrin-mediated adhesion and spreading in immortalized cell lines. In addition, Rac3 has been shown to be expressed differentially during myeloid differentiation (U. Knaus, personal communication). Rac3 null mutant mice have been reported (Cho *et al.*, 2005; Corbetta *et al.*, 2005) and are viable, fertile, and without obvious physical anomalies. One group has reported a mild neurological phenotype (Corbetta *et al.*, 2005). In addition, in a p190 Bcr-abl transgenic mouse model of acute lymphoblastic leukemia, Rac3, but not Rac1or Rac2, is activated and Rac3 deficiency attenuates the development of leukemia in female mice (Cho *et al.*, 2005). Data suggest that in the absence of Rac1 and Rac2, Rac3 can mediate an attenuated myelodysplastic phenotype in mice transplanted with p210 Bcr-abl (see preliminary data). However, no systematic analysis of hematopoiesis has been reported in Rac3-/- mice. Using reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot analysis, Burkhalter and co-workers (2002) have reported that the expression of Rac3 expression is downregulated dramatically during terminal myeloid differentiation. The functional significance of this observation has not been reported. We have demonstrated normal neutrophil differentiation in Rac1-/-; Rac2-/- cells (Filippi *et al.*, 2004; Gu *et al.*, 2003), but observed abnormal myeloid development *in vitro* after transduction of HSC/P with the dominant negative D57NRac2, which most likely inhibits Rac3 in addition to Rac1 and Rac2 (Tao *et al.*, 2002). These data suggest that Rac3 may be important in myelopoiesis, but suffer from the weaknesses of the use of DN mutants, the effects of which are not specific and are determined in part by expression levels. Interestingly, HSC/P expressing D57NRac2 fail to reconstitute hematopoiesis when transplanted into lethally irradiated recipients (Gu *et al.*, 2002).

6. Cdc42 in Hematopoiesis

Cdc42 has been linked for some time with gradient sensing and filopodia (Ridley and Hall, 1992). Until very recently, most work in hematopoietic cells utilized macrophage cell lines or examined the role of Cdc42 in lymphocytes, and interest in lymphocytes is derived in part because of the association of the Cdc42 target WASp in the human immunodeficiency disease of the same name (Symons *et al.*, 1996). In macrophages and myeloid cell lines, DN Cdc42 expression or Cdc42 inhibition in cell lines is associated with a lack of polarization in response to the growth factor/chemotactic factor CSF-1, leading to reduced directed but not random migration (Allen *et al.*, 1998; Srinivasan *et al.*, 2003). In monocytes, either constitutive active or DN Cdc42 expression leads to reduced migration across the endothelium (Weber *et al.*, 1998). In primary T cells, DN Cdc42 reduces chemotaxis in response to SDF-1, a potent chemokine for lymphocytes (del Pozo *et al.*, 1999).

More recent studies have utilized gene-targeted mice. Loss of the Cdc42 GEF PIXα leads to defective G-coupled receptor signaling and PAK activation and reduced migration. Gene-targeted mice deficient in the Cdc42 GAP protein exhibit increased Cdc42 activity in the bone marrow with increased apoptosis in HSC populations. Hematopoietic cells exhibit disorganized actin structure and defective engraftment in stem cell transplant protocols (Wang *et al.*, 2006). Neutrophils from gene-targeted Cdc42-deficient mice show increased random motility but reduced directed migration associated with reduced podosome-like structures at the leading edge of the cells (Szczur *et al.*, 2006). Cdc42-/- neutrophils show increased lateral and tail membrane protrusions. Directed migration appears inhibited by defective p38MAPK activity apparently required for antagonizing these lateral filopodia-like structures. HSC from Cdc42-/- mice show defective migration and adhesion, which is associated with abnormal F-actin assembly, homing, and engraftment/retention in the bone marrow. Cdc42-/- mice show increased numbers of circulating HSC and reduced development of erythrocytes with anemia (Yang *et al.*, 2007a). In contrast to Cdc42 GAP-/- mice, these animals do not show increased apoptosis, but do show abnormalities in cell cycle progression associated with dysregulated p21 and cMyc expression. More recent studies show that Cdc42 regulates the balance between myelopoiesis and erythropoiesis (Yang *et al.*, 2007b). Cdc42-deficient mice developed a fatal myeloproliferative disorder characterized by neutrophilia, myeloid cell proliferation, and infiltration into multiple organs. Early erythroid development was inhibited. Bone marrow of Cdc42-/- mice showed decreased erythroid burst-forming units and erythroid colony-forming units. These changes were associated with upregulation of the

myeloid transcription factor PU.1, C/EBP1α, and Gfi-1 and downregulation of GATA-2.

7. RhoA in Hematopoiesis

The effect of RhoA on hematopoiesis has been less well studied compared with Rac and Cdc42. As noted previously, activation of RhoA leads to stress fiber formation and cell shape changes, although most of these studies have been performed on fibroblasts. In fibroblasts, activation of RhoA has been reported to decrease the expression of Cdk inhibitors and to shorten G1 (Olson *et al.*, 1998). Using the same cell types, inactivation of RhoA has been shown to induce the expression of cyclin D–Cdk4 complexes in early G1 phase and promote a rapid G1/S phase transition (Roovers *et al.*, 2003; Welsh *et al.*, 2001). In mammary gland epithelial cells, transforming growth factor-β-induced activation of RhoA stimulates the nuclear translocation of p160 ROCK, a known target of RhoA, which results in cell cycle arrest by decreasing the activity of Cdc25A phosphatase and decreasing Rb phosphorylation (Bhowmick *et al.*, 2003). Therefore, the effect of RhoA GTPase activity on cell cycle and proliferation appears both cell type and agonist specific.

We have examined the role of RhoA GTPase in hematopoietic stem and progenitor cell functions by expressing DN mutant RhoAN19 in HSC/P via retrovirus-mediated gene transfer (Ghiaur *et al.*, 2006). In contrast with the published role of RhoA in fate determination and differentiation in mesenchymal stem cells (Sordella *et al.*, 2003), inhibition of RhoA activity was associated with a significant enhancement of HSC engraftment and reconstitution *in vivo*. Increased engraftment of HSC expressing RhoAN19 was associated with increased cyclin D1 expression and enhanced proliferation and cell cycle progression of hematopoietic progenitor cells *in vitro*, despite this enhanced engraftment *in vivo*. Consistent with studies reported in fibroblast cells (Hall, 1998), RhoA was essential for normal adhesion and migration of hematopoietic progenitor cells *in vitro*. Decreased activity of RhoA GTPase resulted in defective $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin-mediated adhesion and impaired SDF-1α-directed migration of hematopoietic progenitor cells *in vitro*. These results are surprising given the role of adhesion and migration in HSC engraftment. Taken together, these data suggest that RhoA GTPase plays a crucial role in HSC engraftment, although the mechanism of enhanced engraftment seen with expression of the DN RhoA protein is unclear. In the context of previous reports describing Rac GTPase function in HSC (Cancelas *et al.*, 2005; Gu *et al.*, 2003), these studies suggest that inhibition in Rac activity may enhance mobilization, whereas inhibition of RhoA may augment HSC engraftment.

Additional studies using gene-targeted mice are needed to better clarify the role of RhoA, RhoB, and RhoC in hematopoiesis.

8. RhoGTPase in Human Diseases

8.1. Rac deficiency syndrome

The initial report of Rac2-deficient mice described a phagocytic immunodeficiency syndrome emphasizing neutrophil dysfunctions related to actin cytoskeletal abnormalities. Subsequently, Ambruso *et al.* (2000) and our own group (Williams *et al.*, 2000) reported the identification of a child with serious, life-threatening infections associated with a dominant negative mutation of Rac2 (D57N). The patient exhibited leukocytosis but reduced inflammatory infiltrate in areas of infection. Neutrophils from this patient responded normally with respect to the respiratory burst to phorbol 12-myristate 13-acetate. Normal expression of CD11b, CD11c, and CD18 suggested that the patient did not suffer from leukocyte adhesion deficiency (LAD) or classical chronic granulomatous disease (CGD). However, the patient's neutrophils exhibited decreased chemotaxis in response to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukin-8, reduced rolling on GlyCAM-1 (a ligand for L-selectin), reduced superoxide generation in response to fMLP, mildly reduced phagocytosis, and adhesion to fibrinogen. Thus, the patient appeared clinically to have a phenotype overlapping between LAD and CGD.

At the molecular level, both genomic and cDNA sequencing confirmed the presence of the Asp→Asn mutation at position 57. Genomic sequencing confirmed a mono-allelic change in the gene, and ≈50% of the cloned cDNAs exhibited this mutation. Expression of the mutant protein via retrovirus-mediated gene transfer in normal neutrophils reproduced the cellular phenotype. The mutant protein displayed 10% GTP-binding activity, resulting in a markedly enhanced rate of GTP dissociation and did not respond to GEFs (Gu *et al.*, 2001). When expressed in murine-derived HSC, D57N Rac2 reduced endogenous activities of both Rac1 and Rac2 and led to decreased cell expansion *in vitro* associated with increased apoptosis. Transplantation of transduced bone marrow into lethally irradiated recipients showed a markedly reduced reconstitution of hematopoiesis in mutant-expressing cells over time, consistent with the role of Rac GTPases in marrow engraftment and retention described earlier. Interestingly, prior to successful curative allogeneic transplantation of this patient, his peripheral blood counts were diminishing and he was mildly pancytopenic at the time of marrow ablation in preparation for the transplant.

Taken together, these data suggest that the mutation behaves as a dominant negative mutation, likely by sequestering multiple GEFs in the cell.

This is a highly conserved amino acid in all GTPases and in the Ras superfamily and coordinates the binding of the γ-phosphate to the GTPase. Addition of recombinant Rac2 to cell-free extracts from the patient's neutrophils restored superoxide production, demonstrating the specificity of the molecular mutation in Rac2. This single case is the first reported mutation in humans of a GTPase and provides a fascinating correlation between the basic biology as elucidated in gene targeting models and human disease phenotype. Undoubtedly additional similar cases will become apparent, as many children with recurrent infections and neutrophil dysfunction remain poorly characterized at the molecular level.

8.2. Wiskott–Aldrich disease

The Cdc42 effector protein WASp is defective in the X-linked immunodeficiency disorder Wiskott–Aldrich syndrome (WAS) (Ochs and Thrasher, 2006; Thrasher and Burns, 1999). WASp activation depends on the specific interaction with guanosine triphosphate (GTP)-loaded Cdc42, which is mediated through a Cdc42- and Rac-interactive binding (CRIB) domain (Abdul-Manan *et al.*, 1999; Miki *et al.*, 1998; Rohatgi *et al.*, 1999). WASp is expressed in a hematopoietic-specific fashion. A spectrum of clinical disease is seen that correlates with mutations in specific domains of the WASp protein. Classic WAS patients express no WASp and have severely defective immune function that is characterized by aberrant polarization and directed migration of hematopoietic cells. WASp and the isoform N-WASp activate Arp 2/3, which regulates polymerization of actin from the barbed and branching filaments. WASp-deficient mice have been generated and also show significant hematopoietic defects.

Macrophages and dendritic cells from patients with WAS and from WASp-deficient mice have been shown to be defective in their migratory behavior. Chemotaxis of mutant macrophages in response to macrophage colony-stimulating factor (M-CSF), fMLP, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1a (MIP1 a) has been shown to be abrogated (Badolato *et al.*, 1998; Zicha *et al.*, 1998). WASp-deficient dendritric cells (DCs) exhibit similar abnormalities of cytoskeletal organization, chemotaxis, and migration (Binks *et al.*, 1998). WASp-deficient murine DCs exhibit multiple defects of trafficking *in vivo* after stimulation, including the emigration of Langerhans cells from the skin to secondary lymphoid tissues and the correct localization of DCs within T-cell areas, which correlated with a deficient migratory response of dendritic cells to the chemokines CCL19 and CCL21 (de Noronha *et al.*, 2005; Snapper *et al.*, 2005). It is therefore possible that DC trafficking abnormalities contribute in a significant way to the immune dysregulation observed in WAS and are responsible for the inflammation initiation and eczema development in this disease.

Defects of migration, anchorage, and localization have been defined more recently for other cell lineages, including T and B lymphocytes, neutrophils, and HSC/P. T lymphocytes from patients with WAS respond less well than normal cells *in vitro* to CXCL12 and CCL19 and demonstrate abrogated homing to secondary lymphoid tissue after adoptive transfer *in vivo* (Haddad *et al.*, 2001; Snapper *et al.*, 2005). There is a defect in the localization and function of the immunologic synapse, as WASp is recruited to lipid rafts immediately after the T-cell receptor and CD28 triggering event and is required for the movements of lipid rafts. T cells from WAS patients, lacking WASp, proliferate poorly after TCR/CD28 activation and have impaired capacities to cluster the lipid raft marker GM1 and to upregulate GM1 cell surface expression (Dupre *et al.*, 2002). Interestingly, cells that are deficient for both WASp and Wiskott–Aldrich syndrome protein-interacting protein (WIP) exhibit much more profound deficiencies than either alone, suggesting the existence of some redundancy (Gallego *et al.*, 2006). Similarly, WASp-deficient B lymphocytes have been shown to have marked morphologic abnormalities, defective migration, and adhesion *in vitro* and impaired homing *in vivo* (Westerberg *et al.*, 2005). This defect is likely to contribute to the observed deficiencies of humoral responses to both T-dependent and T-independent antigens and to the marked deficiency of marginal zone B cells in both murine and human spleens (Facchetti *et al.*, 1998; Westerberg *et al.*, 2005). A further example of defective trafficking *in vivo* originates from the observation that carrier female subjects for classic WAS almost universally exhibit nonrandom X-inactivation patterns in $CD34^+$ bone marrow progenitors (Wengler *et al.*, 1995). This implies that WASp is functional within the HSC/P compartment and is consistent with evidence for WASp expression in this cell type in human adult and embryonic hematopoietic stem cells (Marshall *et al.*, 2000; Parolini *et al.*, 1997). Serial stem cell transplantation and competitive repopulation studies in mice have confirmed a selective homing and engraftment advantage for normal HSC, and hematopoiesis established by means of engraftment of chimeric fetal liver populations results in dominance of normal HSC/P over WASp-deficient hematopoiesis (Lacout *et al.*, 2003), suggesting that throughout development, there be preferential establishment of hematopoiesis by normal rather than mutant HSCs due to an intrinsic homing advantage.

Much can be learned from the study of human patients with naturally occurring mutations. Most molecular defects in the WASp gene result in diminished activity, either because aborted protein production or because of intrinsic instability the mutant mRNA or protein. However, some mutants have been shown to display impaired interaction with key regulators. For instance, patients with X-linked thrombocytopenia express lower levels of WASp and have residual immune function (Lemahieu *et al.*, 1999). Some WASp gene defects result in expression of mutant protein with amino

acid substitutions within the Ena/VAS homology 1 domain, predictive of a disturbed interaction with WIP (Volkman *et al.*, 2002). Some clinically relevant mutations have been shown to abolish *in vivo* proper N-WASp localization and actin polymerization (Moreau *et al.*, 2000). Some X-linked neutropenia patients have missense mutations in the Cdc42-binding site of the WASp protein, and a subset of these patients has activating mutations that lead to constitutive activation of the protein. These activating mutations act by preventing autoinhibition of the Cdc42-binding domain of the molecule inducing unregulated actin polymerization and abnormal cytoskeletal structure and dynamics (Ancliff *et al.*, 2006). Interestingly, the phenotype of clinical disease arising from these mutations affecting the Cdc42-binding site is quite unlike that of classical WAS. These mutations lead to myelodysplastic changes in the bone marrow, reduced lymphocyte numbers and function, and increased apoptosis in the myeloid lineage associated with neutropenia and markedly abnormal cytoskeletal structure and dynamics. The mechanism of this defect is unclear but it can be related to abnormalities of cytokinesis affecting the chromosomal separation during mitosis.

8.3. Rac hyperactivation in leukemia

Rac GTPases have been previously implicated in p210-BCR-ABL-mediated transformation (Burridge and Wennerberg, 2004; Harnois *et al.*, 2003; Renshaw *et al.*, 1996; Schwartz, 2004; Sini *et al.*, 2004; Skorski *et al.*, 1998), although the specific role(s) of individual Rac subfamily members in the development of disease *in vivo* has not been defined. Evidence also suggests that Rac3 plays a role in p190-BCR-ABL-mediated ALL, whereas Rac1 and Rac2 do not appear to be hyperactivated in these lymphoma lysates (Cho *et al.*, 2005). This is of particular relevance, as p190-BCR-ABL differs from p210 in potentially important ways as it relates to RhoGTPases. For instance, while p210-BCR-ABL binds to and activates the Rho GTPases, apparently through the Dbl homology domain, p190-BCR-ABL, which lacks this domain, cannot bind to Rho GTPases but can still activate Rac1 and Cdc42 (Harnois *et al.*, 2003) through activation of the GEF Vav1 by BCR-ABL (Bassermann *et al.*, 2002). Rac GTPases have been shown to regulate signaling pathways that are downstream of p210-BCR-ABL (Burridge and Wennerberg, 2004; Schwartz, 2004). Together, these data suggest that Rac GTPases may integrate multiple signaling components of p210-BCR-ABL-activated pathways.

We analyzed whether Rac isoforms were hyperactivated in human chronic phase CML HSC/P. Activation of Rac was determined by p21-activated kinase (PAK) binding domain pull-down assays in isolated $CD34^+$ cells from CML patients. We observed that Rac1, Rac2, and, to a lesser degree, Rac3 were hyperactivated in $CD34^+$ cells purified from peripheral

blood of two CML patients at diagnosis (Thomas *et al.*, 2007). We subsequently utilized a retroviral murine model in Rac gene-targeted BM cells to investigate the importance of Rac GTPase activation in the development and progression of p210-BCR-ABL-mediated MPD. We showed that the combined deficiency of Rac1 and Rac2 significantly attenuates p210-BCR-ABL-induced proliferation *in vitro* and MPD *in vivo*. Attenuation of the disease phenotype is associated with severely diminished p210-BCR-ABL-induced downstream signaling in primary hematopoietic cells. These data are consistent with previous reports of Rac3 activation in p190-BCR-ABL expressing malignant precursor B-lineage lymphoid cells (Cho *et al.*, 2005). We then utilized NSC23766, a small molecule antagonist of Rac activation (Gao *et al.*, 2004), to biochemically and functionally validate Rac as a molecular target in both a relevant animal model and in primary human CML cells *in vitro* and in a xenograft model *in vivo*, including in imatinib-resistant p210-BCR-ABL disease. These data demonstrate that Rac is an important signaling molecule in BCR-ABL-induced transformation and an additional therapeutic target in p210-BCR-ABL-mediated myeloproliferative disease. Additional studies in other chronic and acute myelogenous leukemia may define the role of Rac and other GTPases in both chronic and acute leukemias.

8.4. RhoH and lymphomas

The RhoH/TTF (Translocation Three Four) gene was first identified as a fusion protein containing the LAZ3/BCL6 oncogene as a result of the t(3;4)(q27;p11) translocation in a non-Hodgkin's lymphoma (NHL) cell line (Dallery *et al.*, 1995; Dallery-Prudhomme *et al.*, 1997). A chromosomal alteration involving the RhoH/TTF gene in the t(4;14)(p13;q32) translocation has also been found in another patient with multiple myeloma (Preudhomme *et al.*, 2000). In some cases, RT-PCR analyses of NHL patients have shown deregulated expression of both RhoH and BCL6 genes by promoter exchange between these two genes (Preudhomme *et al.*, 2000). The RhoH gene, along with three other oncogenes (PIM1, MYC, and PAX5), has been found to have a more than 45% mutation rate in human diffuse large B-cell lymphomas (DLBCLs) (Pasqualucci *et al.*, 2001). Mapping analyses demonstrated mutations scattered throughout the 1.6 kb of intron 1 in the RhoH gene in 13 of 28 DLBCLs, suggesting potential effects on the regulation of RhoH gene expression with pathophysiological relevance. Similar aberrant hypermutation in the RhoH gene also occurs in AIDS-related non-Hodgkin lymphomas (Gaidano *et al.*, 2003) and primary central nervous system lymphomas (Montesinos-Rongen *et al.*, 2004). However, it remains unclear whether these mutations translate into abnormal levels of RhoH expression in lymphomas and what physiological contribution hypermutation in the RhoH gene plays in

lymphomagenesis. p53, a tumor suppressor gene, is a key regulator of apoptosis and cell cycle arrest upon DNA damage in many cells. p53 is the most frequently altered tumor suppressor in human solid tumors and is also altered in hematologic malignancies. Interestingly, p53 inactivation is frequent in transformed follicular lymphomas (80%) (Lo Coco *et al.*, 1993) and Burkitt's lymphoma (28%) (Kaneko *et al.*, 1996; Preudhomme *et al.*, 1995), suggesting that the frequency of p53 mutations in NHL may be higher than in other hematopoietic malignancies. Activating mutants of Rac1 cooperate with p53 deficiency to promote primary mouse embryonic fibroblast transformation and/or invasion, suggesting a possible functional cooperation between loss of the p53 gene and Rho GTPase-mediated signaling pathways in tumorigenesis.

The human RhoH/TTF gene encodes a 191 amino acid protein belonging to the Rho GTPase family. The C-terminal tail of RhoH, CKIF, represents a typical CAAX motif present in the entire Ras superfamily of small GTP-binding proteins. Proteins containing this motif will be geranylated if the C-terminal amino acid (X) is leucine (L) or phenylalanine (F). This post-translational modification plays a critical role in the localization of Ras and Rho proteins to the plasma membrane (Kinsella *et al.*, 1991). Biochemical studies showed that RhoH is GTPase deficient and remains constitutively in the active, GTP-bound state (Li *et al.*, 2002b). Interestingly, RhoH and RhoE are naturally GTPase deficient due to the amino acid substitutions at key residues that are highly conserved among all Rho GTPases (Li *et al.*, 2002b). This suggests that in contrast to many other family members, regulation of RhoH and RhoE may depend on the level of the protein expressed in the cells rather than guanine nucleotide cycling. Possible mechanisms for regulating RhoH and RhoE activity may include transcriptional, translational, and post-translational processes, which have not been well studied.

Like Rac2, RhoH is expressed only in the hematopoietic lineages, reportedly predominantly in T- and B-cell lines (Dallery-Prudhomme *et al.*, 1997; Li *et al.*, 2002b). Studies in Jurkat cells showed that RhoH expression is transcriptionally regulated upon stimulation with cytokines. Under physiological conditions, RhoH transcripts are also found differentially expressed in murine Th1 and Th2 T-cell subpopulations (Li *et al.*, 2002b), suggesting that RhoH may play a role in differentiation or function in Th1 and Th2 cells. However, these studies have been limited to lymphocytes and are mainly based on cell lines.

Alteration of RhoH expression experimentally affects proliferation and engraftment of hematopoietic progenitor cells (Gu *et al.*, 2005a) and integrin-mediated adhesion in Jurkat cells (Cherry *et al.*, 2004). We and others have determined major physiological functions of RhoH using gene-targeted mice deficient in the RhoH protein. *RhoH*$^{-/-}$ mice demonstrate impaired TCR-mediated thymocyte positive selection and maturation,

resulting in T-cell deficiency (Dorn *et al.*, 2007; Gu *et al.*, 2006). Loss of RhoH leads to defective CD3ζ phosphorylation, impaired translocation of ZAP-70 to the immunological synapse, and reduced activation of ZAP-70-mediated pathways in thymic and peripheral T cells. Furthermore, proteomic analysis demonstrated RhoH to be a component of TCR signaling via TCR-activated ZAP-70 SH2-mediated interaction with immunoreceptor tyrosine-based activation motifs (ITAMs) in RhoH. *In vivo* reconstitution studies showed that RhoH function in thymopoiesis is dependent on phosphorylation of the ITAMs. These findings suggest that RhoH is a critical regulator of thymocyte development and TCR signaling by mediating recruitment and activation of ZAP-70. While a direct relationship has yet to be ascertained, taken together these experiments suggest that alterations in the expression and/or function of RhoH may play a role in lymphoma formation. Clearly RhoH is an important signaling molecule in T-cell development, although its exact role in T-cell receptor signaling remains to be elucidated.

9. Summary and Perspectives

The development of gene-targeted mice deficient in Rac GTPases and the use of knockout mice to study the role of these important molecular switches have contributed to the understanding of the role of Rho GTPase in normal blood cell development and function and, indeed, have led to the delineation of complex functioning of Rho GTPases in primary cells in physiological settings. This is particularly true with regard to the unique functions of different Rac molecules in HSC/P, which has not previously been studied or appreciated because of the lack of specificity of experimental methods relying on activated or dominant negative mutants and cell lines. To summarize, Rac2 deficiency leads to a variety of cellular phenotypes in hematopoietic cells, including abnormalities in cell adhesion, migration, degranulation, and phagocytosis as a consequence of abnormal F-actin assembly. Surprisingly, Rac2 appears to regulate survival in several cell types via activation of Akt pathways. In contrast, Rac1 regulates both overlapping and unique F-actin functions. and these differences appear, at least in neutrophils, to be because of differences in intracellular localization controlled by sequences in the carboxy-terminal tail and a specific region of the protein not previously implicated in Rac function (Filippi *et al.*, 2004). Rac1 regulates HSC/P cell cycle progression. Significantly, Rac1 is critical to stem cell engraftment and Rac2 is a major determinant of HSC/P retention in the marrow cavity. In addition, Cdc42 and RhoA may play an opposite role in HSC/P homing and engraftment. Thus, it is possible that each Rho family member will be utilized differently in these processes.

Overall, these studies implicate Rac, Cdc42, and RhoA as major regulators of HSC/P engraftment and marrow retention and begin to define the ''intracellular signaling profile of the stem cell niche.'' Indeed, these studies suggest that engraftment and mobilization are separable biochemically and imply that these processes are not ''mirror images'' functionally.

These studies have also raised several unanswered questions. They include what the specific effector pathways (such as STAT, PAK, or WAVE) are downstream of individual Rho GTPases critical for stem cell adhesion, engraftment, and retention, as well as for stem cell transformation. Whether the altered cell adhesion and migration properties of HSC/Ps are directly responsible for the engraftment effect and for the survival/proliferation will also need to be dissected. Finally, the specificity of upstream guanine exchange factors coupling to the upstream stimuli of SDF1a, SCF, or integrins in the activation of Rho GTPases will need to be determined in hematopoietic cells.

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REVIEW

Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis

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Chronic myelogenous leukemia (CML) is a malignant disease characterized by expression of p210-BCR-ABL, the product of the Philadelphia chromosome. Survival of CML patients has been significantly improved with the introduction of tyrosine kinase inhibitors that induce long-term hematologic remissions. However, mounting evidence indicates that the use of a single tyrosine kinase inhibitor does not cure this disease due to the persistence of p210-BCR-ABL at the molecular level or the acquired resistance in the stem cell compartment to individual inhibitors. We have recently shown in a murine model that deficiency of the Rho GTPases Rac1 and Rac2 significantly reduces p210-BCR-ABL-mediated proliferation *in vitro* and myeloproliferative disease *in vivo*, suggesting Rac as a potential therapeutic target in p210-BCR-ABL-induced disease. This target has been further validated using a first-generation Rac-specific small molecule inhibitor. In this review we describe the role of Rac GTPases in p210-BCR-ABL-induced leukemogenesis and explore the possibility of combinatorial therapies that include tyrosine kinase inhibitor(s) and Rac GTPase inhibitors in the treatment of CML.
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Introduction

The p210-BCR-ABL fusion protein that is generated from a reciprocal translocation between the breakpoint-cluster region (BCR) gene on Chromosome 22 and the Abelson leukemia (ABL) gene on Chromosome 9 is necessary and sufficient for the development of chronic myelogenous leukemia (CML).[1,2] Although allogeneic stem cell transplantation is a curative therapy for the treatment of CML, most patients lack suitable donors or are not eligible for transplant due to advanced age.[3–6] The development of imatinib mesylate, a tyrosine kinase inhibitor that has been shown to induce complete hematologic and cytogenetic responses in many patients, has provided an effective means of treatment of CML and has rejuvenated the field of rationalized drug design.[7] Imatinib targets the abnormal kinase activity of CML blasts and induces apoptosis of p210-BCR-ABL$^+$ cells.[8,9] However, in a proportion of patients the persistence of p210-BCR-ABL$^+$ cells or the development of p210-BCR-ABL kinase mutants that confer resistance to imatinib have been demonstrated.[10,11] While a second generation of tyrosine kinase inhibitors including nilotinib and dasatinib are effective at inhibiting the activities of most imatinib-resistant p210-BCR-ABL mutants, the use of sequential Abl kinase inhibitor therapy has been shown to select for compound mutations that confer resistance to both drugs and increase p210-BCR-ABL oncogenicity,[12] suggesting that other signaling components downstream of p210-BCR-ABL should be considered as potential therapeutic targets.

The Rac subfamily of Rho GTPases comprising the highly related mammalian proteins Rac1, Rac2 and Rac3 has previously been implicated in p210-BCR-ABL-mediated transformation using cell lines and in acute myelogenous leukemia (AML) cell migration dependent on vascular endothelial growth factor paracrine stimulation.[13] Rac1, in particular, has been identified as an important downstream component of BCR-ABL signaling, suggesting Rac GTPases as possible molecular targets for interrupting abnormal signaling in CML blasts.[14–19] Our findings that the combinatorial loss of Rac1 and Rac2 significantly attenuates p210-BCR-ABL-induced proliferation *in vitro* and myeloproliferative disease (MPD) *in vivo* provide additional genetic evidence that the Rac GTPases may be attractive therapeutic targets in p210-BCR-ABL-mediated MPD.[20] These genetic data were further substantiated experimentally by use of NSC23766, a first-generation small molecule inhibitor that specifically blocks activation of the Rac GTPases. While our data confirm that Rac GTPases are candidate therapeutic targets in p210-BCR-ABL-mediated disease, a number of questions remain regarding the role of Rac and other Rho GTPases in p210-BCR-ABL-induced leukemogenesis.

Relationship between Rac GTPases and p210-BCR-ABL in CML

The Rac subfamily of Rho GTPases has been implicated in a variety of different cellular functions, including adhesion, migration, actin assembly, transcription activation, cell cycle progression and cell survival (reviewed in Blanchard[21]). Similar to other Ras-related GTPases, Rac GTPases cycle between inactive, GDP-bound and active, GTP-bound conformations to transduce signals to effector proteins that mediate a multitude of cellular responses. Three structurally related proteins, Rac1, Rac2 and Rac3, have been identified. While Rac1 and Rac3 are ubiquitously expressed, expression of Rac2 is restricted to hematopoietic tissues. Thus, hematopoietic cells are unique in expressing all three Rac proteins. Our laboratory has previously shown that Rac1 and Rac2 are essential for the regulation of multiple hematopoietic stem cell functions with unique as well as overlapping roles, including adhesion, migration, proliferation and apoptosis.[22] In effecting these responses, Rac GTPases

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have been shown to activate signaling molecules that coincide with known downstream targets of p210-BCR-ABL,[18,19] such as the Ras/MAP kinases (ERK, p38 and JNK), phosphatidylinositol-3-kinase (PI3K)/Akt, Bcl-X_L and focal adhesion kinase (FAK).

These earlier observations highlight a possible relationship between Rac GTPases and p210-BCR-ABL, although the specific role(s) of the individual Rac subfamily members in the development of disease *in vivo* have not previously been defined. Skorski *et al*.[16] showed that activation of Rac GTPases is enhanced in 32Dcl3 myeloid precursor cells ectopically expressing p210-BCR-ABL. Additionally, survival of mice injected with 32Dcl3 cells co-expressing p210-BCR-ABL and a dominant-negative N17Rac mutant was markedly extended, compared to mice transplanted with 32Dcl3 cells expressing p210-BCR-ABL alone.[16] Harnois *et al*.[17] showed that the p210-BCR-ABL fusion protein forms a stable complex with Rac1, Rac2, and other RhoGTPases including RhoA and Cdc42 and may directly activate these Rho GTPases through the Dbl homology domain of Bcr. Conversely, these Rho GTPases may be activated by p210-BCR-ABL through the recruitment of Vav1,[17] a hematopoietic-specific guanine nucleotide exchange factor (GEF) that is crucial for Rac activation in lymphoid[23] and other hematopoietic cells. Sini *et al*.[14] have described activation of Rac by Abl-induced tyrosine phosphorylation of Sos-1, which could be inhibited by genetic or pharmacological inhibition of Abl. Additionally, p210-BCR-ABL has previously been shown to display a Rac-dependent induction in transformation-associated changes in cytoskeletal functions such as actin assembly, migration and adhesion, all known functions of Rho GTPases, particularly Rac.[18,19] Finally, Diaz-Blanco *et al*.[24] have recently shown that Rac1 and Rac2 were significantly upregulated in CD34+ human chronic phase CML bone marrow (BM) cells. These and other data imply that p210-BCR-ABL interacts directly and/or indirectly with Rac, Rho and Cdc42 to activate these GTPases in cell lines.

In support of this postulated involvement of Rac GTPases in p210-BCR-ABL-mediated disease, we have demonstrated hyperactivation of Rac1 and Rac2 and, to a lesser extent, Rac3 in hematopoietic stem cells and progenitors (HSC/P) isolated from chronic phase CML patients. These data confirm that Rac GTPases are abnormally activated in chronic phase disease. Experimentally, Rac GTPases were also shown to be hyperactivated in primary murine BM cells expressing p210-BCR-ABL after retrovirus-mediated gene transfer.[20] To determine the importance of the individual Rac GTPases in the development of p210-BCR-ABL-mediated CML, we employed an *in vivo* retroviral murine model of hematopoietic stem cell transformation combined with the use of BM cells from gene-targeted mice to effect deletion of Rac1 alone, Rac2 alone and Rac1 in combination with Rac2. As originally described,[1] this model has demonstrated that expression of p210-BCR-ABL in murine HSC/P can induce an MPD, including the development of leukocytosis, splenomegaly, extramedullary hematopoiesis in the liver and pulmonary hemorrhage due to extensive granulocyte infiltration in the lung. In our reported studies, while the median survival of p210-BCR-ABL-expressing wild-type (WT) and Rac1-deficient mice was 23 and 22 days, respectively, the median survival of p210-BCR-ABL-expressing Rac2-deficient mice was significantly increased to 43 days, and the median survival of p210-BCR-ABL-expressing Rac1/Rac2-deficient mice was even more strikingly increased to 92 days. This result suggests that individual Rac GTPases play unique roles in p210-BCR-ABL-mediated leukemogenesis, as has been described for normal HSC/P functions.[22,25]

Using this genetic approach, we also monitored the disease phenotype of the p210-BCR-ABL-expressing WT and Rac-deficient animals. Expression of p210-BCR-ABL in WT, Rac1- and Rac2-deficient HSC/P led to the development of oligoclonal myeloid-lineage leukemias. Expression of p210-BCR-ABL in Rac1/Rac2-deficient HSC/P led to altered disease phenotype, with mice showing oligoclonal leukemias of myeloid, lymphoid or bi-lineage immunophenotypes, suggesting that Rac1 and Rac2 are critical for transformation and MPD development *in vivo*. The mechanism of these differences in disease phenotypes is still being investigated but could be related to alterations in downstream signaling pathways in the absence of Rac1 and Rac2 and/or compensatory alterations in the activity of Rac3.

Loss of Rac GTPases alters the signaling cascades activated by p210-BCR-ABL

The results presented above suggest that Rac1 and Rac2 play an important role in the development of p210-BCR-ABL-mediated MPD, but in the absence of these GTPases, expression of p210-BCR-ABL can lead to the eventual progression of phenotypically altered disease via unspecified downstream signaling components. What is the mechanism by which p210-BCR-ABL mediates disease in the absence of Rac1 and Rac2? To answer this question, we first analyzed the status of Rac3 activation in splenocytes harvested from leukemic Rac1/Rac2-deficient animals. Rac3 is the third member of the Rac subfamily of Rho GTPases that was originally discovered by screening the p210-BCR-ABL-expressing erythroid blastic-phase CML cell line K562.[26] Rac3 activation has been demonstrated in p190-BCR-ABL-expressing malignant precursor B-lineage lymphoblasts[27] and is associated with the invasive phenotype of breast carcinomas,[28,29] suggesting that Rac3 hyperactivation could play a specific role in cancer development and invasiveness. We demonstrated that Rac3 was hyperactivated in p210-BCR-ABL-expressing leukemic animals in the absence of Rac1 and Rac2.[20] These data, along with the observed differences in survival mediated by Rac1- versus Rac2-deficient HSC, support the hypothesis that individual Rac GTPases play unique roles in the development of p210-BCR-ABL-mediated disease. Studies are underway to further explore the specific roles of each Rac GTPase in disease evolution and phenotype.

Mice that express p210-BCR-ABL with a point mutation in the ATP-binding site of ABL do not develop leukemia.[30] This indicates that ABL kinase activity is required for p210-BCR-ABL-induced transformation. However, the p210-BCR-ABL fusion protein is also composed of several structural domains that play distinct roles in cell signaling. Phosphorylation of Bcr at tyrosine 177 recruits Grb2/Gab2 and Sos, which results in Ras, ERK, JNK and p38 MAP kinase activation.[31,32] Phosphorylation of Bcr at tyrosine 177 also leads to the recruitment of SHP2 and PI3K/Akt.[33] Ras may be activated by two additional substrates of p210-BCR-ABL, the adapter molecules Shc and CrkL.[34,35] The actin-binding domain and the C-terminal domain of ABL, while not necessary for p210-BCR-ABL-mediated leukemogenesis experimentally, may contribute to the malignant behavior of p210-BCR-ABL leukemic blasts.[36] As mentioned previously, the role of a Dbl homology domain present in p210-BCR-ABL but not in a shorter form of BCR-ABL (p190-BCR-ABL) remains controversial. This domain may mediate Rac activation[17] that has been demonstrated to be necessary for full Ras-mediated transformation.[16] Finally, the Src homology domains SH2 and SH3 of p210-BCR-ABL bind to the Src family kinase member Hck and can phosphorylate signal transducer and activator of

transcription 5 (STAT5) independently of Janus kinase activation.[37] These known signaling functions of the fusion protein suggest that p210-BCR-ABL-mediated signals may converge on Rac through several pathways such as Ras/MAPK (ERK, p38 and JNK), PI3K/Akt, Bcl-x_L and FAK to alter proliferation and survival.

We analyzed activation of ERK, JNK, p38, Akt, STAT5 and CrkL in splenocytes harvested from p210-BCR-ABL-expressing WT, Rac1-deficient, Rac2-deficient and Rac1/Rac2-deficient animals. Increased baseline phosphorylation of each of these signaling components was apparent in cells derived from leukemic WT animals, as well as Rac1-deficient leukemic animals. However, activation of downstream pathways including ERK, JNK, p38 and Akt was attenuated in Rac2-deficient leukemic cells and almost completely abrogated in the Rac1/Rac2-deficient cells, correlating with the overall survival that was observed in animals from each of these genotypes. The decreased activation of downstream pathways was not due to decreased ABL tyrosine kinase activity, as autophosphorylation of p210-BCR-ABL was still noted in these cells.[20] STAT5 phosphorylation also was still detectable in leukemic cells regardless of the presence or absence of Rac1 and Rac2 GTPase activity. These data suggest that STAT5 may be the crucial signaling component for leukemia development in Rac1/Rac2-deficient HSC/P.

Surprisingly, activation of CrkL, which has been suggested to be an effector that binds directly to p210-BCR-ABL,[34] was decreased in Rac2-deficient and practically abrogated in Rac1/Rac2-deficient leukemias. CrkL activation has recently been reported to be dependent on a large multimeric protein complex that contains at least PI3K, docking protein 2 (DOK2), CrkL, Vav and Rac.[38,39] Thus, our data support a hypothesis that Rac participates in the activation of CrkL in the context of a multiprotein complex.

Putative role of STAT5 in the development of Rac3-mediated MPD

As mentioned previously, Rac3 was found to be hyperactivated in Rac1/Rac2-deficient leukemic animals, suggesting that this GTPase may be important in the eventual development of disease. These results imply that either the individual Rac GTPases play specific roles in the development of p210-BCR-ABL-mediated disease or the combinatorial loss of Rac expression modulates the disease phenotype. Interestingly, STAT5 activation was also apparent in Rac1/Rac2-deficient leukemic animals, suggesting that disease development also may be modulated by this protein.

STAT5 activation has been shown to be pivotal in myeloid differentiation[40] and multiple groups have demonstrated a critical role of STAT5 in the pathogenesis of CML,[41–45] but STAT proteins in leukemic transformation remain highly controversial. The physical interaction of p210-BCR-ABL and STAT5 was delineated by Nieborowska-Skorska *et al*.[46] using retroviral expression of BCR-ABL mutants in 32Dc13 cells. They showed that deletion of the SH2 domain accompanied by a point mutation in the SH3 domain of p210-BCR-ABL abolished STAT5 activation, as did deletion of both the SH2 and SH3 domains. Nieborowska-Skorska *et al*.[46] also demonstrated that cells expressing these STAT5 activation-deficient p210-BCR-ABL mutants were more apoptotic than cells expressing unmutated protein. Additionally, a constitutively active STAT5 mutant was able to rescue cells expressing STAT5-deficient BCR-ABL mutants from apoptosis while 32Dcl3 cells co-expressing a dominant-negative STAT5 mutant and BCR-ABL still underwent apoptosis, confirming the protective effect of STAT5.[46]

Sillaber *et al*.[47] demonstrated that inducible expression of a truncated STAT5 protein (ΔSTAT5) could dimerize with endogenous STAT5 and inhibit STAT5-induced gene transcription and growth in p210-BCR-ABL-expressing Ba/F3 hematopoietic cells, suggesting that STAT5 activation was responsible for most of the cell growth induced by p210-BCR-ABL. Expression of ΔSTAT5 resulted in inhibition of STAT5-induced transcription and a significant reduction in cell growth due to decreased cell viability and greater cell sensitivity to cytotoxic agents such as hydroxyurea and cytarabine.[47] Ba/F3 cells transfected with a vector expressing the Y177F mutant of BCR-ABL exhibited decreased tyrosine phosphorylation and activation of STAT1 and STAT5, compared with transfectants expressing wild-type BCR-ABL, suggesting that phosphorylation of Tyr-177 may be important for the activation of STAT signaling pathways by BCR-ABL. Tyrosine 177 of BCR-ABL has previously been shown as critical for binding to the adaptor protein GRB2, which mediates Ras/MAP kinase activation, suggesting that STAT5 activation may depend on Ras/Rac activation. Finally, STAT5 activation mediated by autocrine secretion of granulocyte-macrophage colony-stimulating factor has recently been shown to be responsible for the outgrowth of imatinib-resistant CML,[48] implying that STAT5 may be an alternative escape pathway by which leukemic cells circumvent tyrosine kinase inhibition.

Conversely, other studies suggest that STAT5 is not important in the pathogenesis of CML. Specifically, lethally irradiated mice transplanted with p210-BCR-ABL-expressing STAT5a/b N-terminal deletion mutant (Stat5a/b$^{\Delta N/\Delta N}$) cells developed disease as rapidly as mice injected with p210-BCR-ABL-expressing WT cells, suggesting that STAT5 is not essential for the development of p210-BCR-ABL-mediated disease.[49] Similar to our findings with Rac1-/Rac2-deficient BM, the majority of the p210-BCR-ABL-expressing WT mice developed myeloid lineage leukemias, while the p210-BCR-ABL-expressing STAT5a/b N-terminal deletion mutant mice had either myeloid, lymphoid or bilineage leukemias.[49] Interestingly, when the entire Stat5a/b gene locus was deleted, p185-BCR-ABL-expressing Stat5a/b$^{null/null}$ cells were resistant to transformation and did not induce lymphoid leukemia development in mice,[50] suggesting that STAT5 is critical for the development of p185-BCR-ABL-mediated disease. Whether p210-BCR-ABL-expression in the absence of Stat5 (that is, in Stat5a/b$^{null/null}$ cells) can modulate the development of MPD is unknown, but represents an intriguing question. Due to these discrepancies, the role of STAT5 in p210-BCR-ABL-mediated disease needs to be further characterized.

Compensatory hyperactivation of Rac3 in the absence of Rac1 and Rac2 may be responsible for the eventual disease development that is observed. The question now remains whether this Rac3-mediated disease is due to activation of the STAT5 signaling cascade.

Rac GTPases as targets for p210-BCR-ABL-mediated CML therapy

On the basis of these genetic data, we examined the effect of NSC23766 on p210-BCR-ABL-induced transformation. NSC23766 is a first-generation, Rac-specific small molecule inhibitor[51] that was developed based on the GEF-Rac1 GTPase complex and computer-assisted virtual screening. NSC23766 was found to fit into a shallow surface groove of Rac1 that has been shown to be critical for GEF specification. In published

studies, NSC23766 was shown to effectively inhibit Rac protein binding and activation by the Rac-specific GEFs TrioN or Tiam1 in a dose-dependent manner. In contrast, NSC23766 did not interfere with the binding or activation of Cdc42 or RhoA by their respective GEFs. In cells, NSC23766 effectively blocked serum- or platelet-derived growth factor-induced Rac1 activation and lamellipodia formation, but did not affect endogenous Cdc42 or RhoA activity. NSC23766 reduced growth stimulated by the Rac-GEFs Trio or Tiam1, but not proliferation stimulated by the promiscuous Rho/Cdc42 GEFs such as Dbl, Lbc, intersectin or a constitutively active Rac1 mutant. Importantly, NSC23766 suppressed Trio, Tiam1 or Ras-induced cell transformation and was shown to attenuate solid tumor cell line transformation and invasion. When human prostate cancer PC-3 cells were treated with NSC23766, Rac1 activity was downregulated, and proliferation, anchorage independent growth and invasion phenotypes that require endogenous Rac1 activity were inhibited.[51]

We have previously shown that retention of murine HSC/P is a Rac-dependent function.[22,25,52] On the basis of initial observations of NSC23766 inhibition of Rac activation, we subsequently demonstrated mobilization of HSC/P following a single *in vivo* dose of the compound.[25] Compared with phosphate-buffered saline (PBS)-treated controls, the Rac inhibitor induced an ~2-fold increase in circulating progenitors at 6 h after injection. The mobilization of HSC/P by NSC23766 was dose-dependent and reversible, with the number of circulating HSC/P returning to normal values within 24 h post injection. To determine if the effect of the inhibitor was associated with specific inhibition of Rac1 and Rac2, we incubated BM lineage negative/c-kit$^+$ cells with stromal-derived factor-1α and NSC23766. At a dose as low as 10 μM, activation of both Rac1 and Rac2 was inhibited.[25] We further found that NSC23766 was effective in suppressing downstream Rac signaling, as measured by the effect on the phosphorylation status of p21-activated kinase-1, a known effector of Rac. The inhibitor appeared to be both reversible and nontoxic *in vivo* when administered at a dose of 2.5 mg per kg daily for a period of 65 days.[25] Thus, NSC23766 constitutes a Rac-specific small molecule inhibitor that is capable of reversing cancer cell phenotypes associated with Rac deregulation and blocking Rac activation in HSC/P *in vivo*.

To validate Rac GTPases as antileukemic targets in p210-BCR-ABL-induced disease, we incubated p210-BCR-ABL-expressing murine cells with increasing concentrations of NSC23766.[20] NSC23766 potently inhibited the growth of these cells and also suppressed proliferation of Rac1/Rac2-deficient cells harvested from p210-BCR-ABL-expressing mice, further supporting the role of Rac3 hyperactivation in disease development. In addition, NSC23766 inhibited proliferation of cells expressing the tyrosine-kinase inhibitor-resistant p210-BCR-ABL-T315I mutation. In the presence of both NSC23766 and imatinib, proliferation of these cells was inhibited by >90%. Treatment with NSC23766 also led to selective killing of human CML blast crisis HSC/P with limited toxicity on normal murine or human HSC/P *in vitro*.

We then determined the effect of NSC23766 *in vivo* using our retroviral transduction and transplantation model. Mice in which NSC23766 was continually administered survived significantly longer than PBS-treated mice. NSC23766 reversed the CML survival/growth in an *in vivo* model of human CML disease after transplantation of chronic-phase purified CD34+ cells in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice.[20] These results further validate Rac as a candidate target in p210-BCR-ABL-mediated disease.

Could targeting Rac GTPases be useful for inducing leukemia stem cell egression from the nurturing leukemic stem cell niche?

Recently, the 'cancer stem cell' hypothesis, which attempts to explain the presence of 'residual' therapy-resistant cancer cells in patients and in related animal models, has attracted much attention.[53] The theory suggests that cancer may arise from a rare population of putative cancer stem cells. Leukemia stem cells (LSCs), which share characteristics with normal HSCs but initiate disease instead of supporting normal hematopoiesis, have been demonstrated in several leukemias, most prominently CML.[54] Like normal HSCs, LSCs are thought to reside in the BM niche, although the nature of LSC interaction with the supporting BM microenvironment, where leukemia presumably arises, remains unclear.[54] Recent studies in a human AML mouse model and a CML mouse model using anti-CD44 antibody to disrupt potential LSC interactions with the BM niche have provided strong evidence that LSCs depend upon interactions within a specific niche.[55,56] The fact that targeting the LSC-expressed cell surface molecule CD44 by monoclonal antibodies effectively suppressed both AML and CML leukemia progression, and induced LSC differentiation raises the possibility that the LSC–niche interaction could be a valid drug target for more effective eradication of leukemia. Since Rac activities are dysregulated in CML and Rac is known to be a central regulator of HSC adhesion, migration and interaction with the BM niche,[22,25] it is possible that Rac targeting by specific inhibitors could transiently mobilize LSCs for therapeutic benefits. As mentioned above, our group has shown previously that Rac1$^{-/-}$;Rac2$^{-/-}$ HSCs demonstrate defective adhesion, migration and lodging in the BM endosteum, and administration of the Rac activation inhibitor NSC23766 induces stem cell mobilization in a dose-dependent manner. It is thus an attractive proposal that future strategies of Rac targeting may be adopted in a similar fashion to induce egression of CML stem cells from the BM niche.

What type of CML patients may benefit from Rac inhibitor therapies?

Since multiple pathways, directly dependent or independent of BCR-ABL expression, are activated in CML during the evolution of disease, we postulate that Rac inhibition in conjunction with ABL tyrosine kinase domain inhibitors may represent a novel method of combined therapy early in the disease.

Levels of *BCR-ABL* mRNA,[57–59] protein[60] or phosphoprotein[61] increase during disease progression. This may be related to the higher levels of expression of BCR-ABL in CD34+ CML cells compared with more differentiated myeloid cells, and the fact that some patients even in complete cytogenetic response display a persistent population of HSC/P that express high levels of BCR-ABL.[62] High levels of expression of BCR/ABL are probably responsible for the fact that intracellular drug levels may be insufficient to reach the degree of kinase inhibition required to induce cell death in HSC/P.[63] Whether there is correlation between BAC-ABL expression and Rac activation is not yet known, but it is quite possible that a BCR-ABL gain of function translates into higher Rac activation. We found that Rac1, Rac2 and to a lesser extent, Rac3 are hyperactivated in CD34+ human chronic phase CML cells,[20] and a Rac-specific small molecule inhibitor significantly reduced the clonogenicity of blastic phase CML granulo-macrophage progenitors, and *in vivo* it significantly reduced the leukemic burden in NOD/SCID

mice transplanted with chronic-phase CML CD34+ cells. Altogether this suggests that Rac inhibition may indeed impair leukemic growth in very different phases of the disease. This is even more relevant when chronic phase CML is believed to derive from HSC with engraftment and self-renewal ability, while blastic phase CML is believed not to derive from HSC but from granulo-macrophage progenitors.[64] The mechanisms of disease persistence in patients treated with tyrosine kinase inhibitors appear to be related to both BCR-ABL-dependent and -independent pathways.[65] Among the BCR-ABL-dependent pathways, the appearance of mutations in the kinase domain that confer high resistance (reviewed in Melo and Barnes[66]), or even moderate degrees of resistance to tyrosine kinase inhibitors[67] and the maintenance of high-expressing HSC/P even in patients in complete cytogenetic response have been shown to be responsible for disease persistence.

Among the BCR-ABL-independent pathways, the overexpression of drug transporters which are likely to influence intracellular levels of tyrosine kinase inhibitors and the simultaneous signaling through cytokine receptor-dependent pathways, in cells that still respond to cytokine stimulation, have been cited.[65] We are unable to confirm whether NSC23766 chemical conformation[51] is an adequate substrate for drug transporters, but since Rac activation is central in many of the key signaling pathways of HSC/P, Rac inhibition may also impair BCR-ABL-independent pathways and represent a valid adjuvant in the therapy of CML. In addition, since Rac appears likely to integrate multiple pathways downstream of BCR-ABL transforming activities, Rac inhibition combined with ABL tyrosine kinase domain inhibitors may represent a novel method of combined therapy early in the disease.

Summary and model of disease development

Rac GTPases appear to play a critical role in the development of leukemogenesis associated with p210-BCR-ABL expression and represent novel targets for therapeutic intervention, as depicted in the model shown in Figure 1. The p210-BCR-ABL fusion protein may activate Rac GTPases either directly through the Dbl homology domain of Bcr or via recruitment of the Vav1 GEF. In addition, loss of Rac expression may inhibit the formation of a large multimeric protein complex containing PI3K, DOK2, CrkL and Vav, thus inhibiting CrkL phosphorylation. Downregulation of Rac activation leads to almost complete abrogation of MAP kinase and PI3K signaling pathways, suggesting that Rac GTPases are required for activation of multiple p210-BCR-ABL-mediated signaling cascades.

Our results also suggest that STAT5 activation is maintained in the absence of Rac1 and Rac2. We speculate that Rac1/Rac2 activation may be a key for p210-BCR-ABL-induced leukemogenesis in this setting. While Rac1 and Rac2 isoforms activate multiple pathways, compensatory Rac3 activation may be responsible for signaling downstream of p210-BCR-ABL and inducing leukemogenesis in the absence of Rac1 and Rac2.

NSC23766, an inhibitor of all three isoforms of Rac that are expressed by hematopoietic cells, induces significant regression of murine p210-BCR-ABL-induced leukemias and human CML in xenogeneic grafts, demonstrating that intervention of Rac activation is a new tool in treating p210-BCR-ABL-induced leukemias. The finding that a single dose of the Rac inhibitor induces mobilization of HSC/P[25] raises the possibility that NSC23766 could also mobilize leukemic stem cells from their niche, thus inhibiting the stem cell properties characteristic of these cells. These results suggest that the Rac GTPases may prove to be useful therapeutically by targeting alternative signaling pathways, which may be responsible for resistance and relapse in CML.

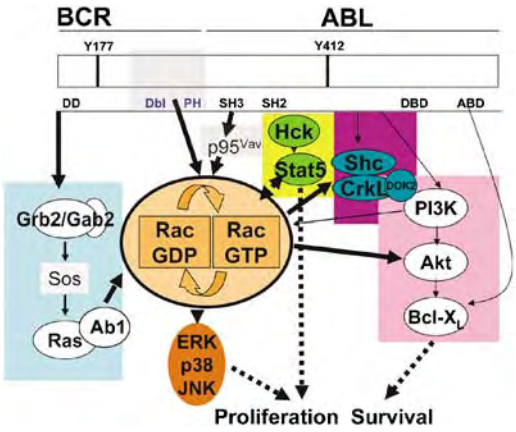

Figure 1 Model of activation of Rac GTPases in BCR-ABL-induced leukemogenesis. The various pathways of activation downstream of p210-BCR-ABL are indicated with different color codes. Gray-shaded areas indicate molecules or domains with guanine nucleotide exchange factor (GEF) activity. The Dbl and pleckstrin homology (Dbl and PH) domains, only present in p210-BCR-ABL, activate Rho GTPases directly. The SH3 domain in both p190- and p210-BCR-ABL activates p95Vav (Vav1). Y177/Y412, tyrosine residues that can be phosphorylated; Dbl, Rac GTPase exchange factor; SH2/SH3, Src homology domains; DD; dimerization domain; DBD, DNA-binding domain; ABD, actin-binding domain.

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